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Abstract

Title of Dissertation:

Biology and ecology of sand flies (Diptera: Psychodidae) in the Middle East, with special emphasis on *Phlebotomus papatasi* and *Phlebotomus alexandri*

Michelle Gayle Colacicco-Mayhugh, Doctor of Philosophy, 2009

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The overall goal of the research presented here is to further the understanding of sand fly biology and ecology in the Middle East region. In order to accomplish this goal, four studies were developed.

The first study examined the effect of climate on sand fly activity. Sand fly collection and meteorological data from Tallil, Iraq from 2003 – 2004 were used in this multivariate analysis. Moon illumination, wind speed, and percent sky cover were inversely related to collections. Temperature was positively correlated. Sand fly activity at Tallil was greatest on warm, clear nights with low wind speed.

In the second study, a niche model of *Phlebotomus papatasi* and *P. alexandri* distribution was developed. The niche modeling program Maxent version 3.2.1 was used to develop predictive models of the distribution of *P. papatasi* and *P. alexandri* across the Middle East. Elevation, land cover, and Worldclim bioclimatic variables were entered into the model. The most influential environmental variable was land cover. The models developed produced maps indicating areas that have a higher probability for the presence of *P. papatasi* and *P. alexandri*.

The third study examined the relationship between *P. papatasi* populations and the normalized difference vegetation index (NDVI), obtained from satellite imagery. NDVI values for October 2004 – September 2005 were compared to sand fly collection data from U.S. military entomologists in Iraq from April – May 2005. There were significant correlations between the May and September collection data and the NDVI values in the preceding winter months. While this indicates there may be some relationship, further research is required to fully evaluate the nature of this relationship.

In the fourth study, *P. alexandri* specimens from Turkey and Iraq were examined to determine if there is a genetic difference between the populations. Cytochrome b (cytb) of the mitochondrial DNA (mtDNA) and the second internal transcribed spacer (ITS2) of the ribosomal DNA (rDNA) were examined in this study. The populations from Turkey and Iraq are different, but there is no difference between populations in Iraq. Further work if required to determine if populations of this species are significantly different across its range.

Biology and ecology of sand flies (Diptera: Psychodidae) in the Middle East, with special emphasis on *Phlebotomus papatasi* and *Phlebotomus alexandri*

By

Michelle Gayle Colacicco-Mayhugh

Dissertation submitted to the Faculty of the Department of Preventive Medicine and Biometrics Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy

2009

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As much of this work relies heavily on the sand fly collections performed in Operations Iraqi and Enduring Freedom, I am tremendously indebted to all military entomologists and preventive medicine personnel who played a role in those collections. In addition, Ms. Nancy Scott at CHPPM-Europe went out of her way to ensure that the flies that I needed arrived in a timely manner.

I would also like to thank Dr. Katie Swanson for her support and friendship throughout this process. Her willingness to listen to me vent helped get me through the days where it seemed as if I would never finish this project.

I am tremendously indebted to my family. My son, Michael, was 12 weeks old when I started this program and is 4 years old as I finish. My daughter, Caitlin, was born while I was pursuing my degree. Neither of them has any idea of what they have been forced to sacrifice during this process. I look forward to being able to have much more "Mommy time" for them. My husband, Chris, helped me summon strength when I

thought I had no more to give, gave me focus when I lost my own, and forced me to take time for myself. Without his love and support I would not have made it through this program.

Finally, my parents have been a crucial component of this endeavor. They raised me with a strong sense of self and a belief that I could accomplish anything I set my mind to. Without that, I never would have been in a position to pursue this degree. In addition, they went out of their way on numerous occasions to help take care of Michael and Caitlin when Chris was deployed to Iraq and I was trying to wrap up my research. For that, I am absolutely grateful.

Dedication

For Ruby Hager, Ann Colacicco, and Gayle Colacicco:

Thank you for teaching a little girl that her options were boundless.

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Chapter 1

Introduction

Sand Fly Taxonomy

The family Psychodidae (Order Diptera) contains five subfamilies: Bruchmomyiinae, Phlebotominae, Psychodinae, Sycorinae, and Trichomyiinae. The sand flies belong to the subfamily Phlebotominae. This subfamily is comprised of six genera, three in the Old World (*Chinius, Phlebotomus*, and *Sergentomyia*) and three in the New World (*Brumptomyia, Lutzomyia*, and *Warileya*). *Phlebotomus* contains ten subgenera: *Spelaeophlebotomus, Idiophlebotomus, Australophlebotomus, Phlebotomus, Paraphlebotomus, Synphlebotomus, Larroussius, Adlerius, Euphlebotomus,* and *Anaphlebotomus* (Lewis et al. 1977, Lewis 1982, Lane 1986, Rispail and Leger 1998a).

Phlebotomus papatasi is one of four members of the subgenus *Phlebotomus* (along with *P. duboscqi, P. salehi*, and *P. bergeroti*). This species was the first sand fly identified and was named *Bibio papatasi* by Johann Anton Scopoli in 1786 (Lewis 1982). The specific name derives from the word " pappataci," meaning silent gorger (Lewis 1982).

Phlebotomus alexandri is a member of the subgenus *Paraphlebotomus*. It was originally described by Newstead in 1920 and classified as *P. sergenti* var. (Lewis 1982). In 1948, Sinton re-classified it as *P. sergenti* var. *alexandri* (Lewis 1982). In 1968, it was elevated to species rank (Lewis 1982).

Medical Importance

Sand flies in the genus *Phlebotomus* are responsible for vectoring Leishmaniasis and sand fly fever. *Phlebotomus papatasi* is a confirmed vector of sand fly fever and

cutaneous leishmaniasis, caused by *L. major* (Ashford 2001a, b). *Phlebotomus alexandri* is a confirmed vector of *L. donovani* in western China (Guan et al. 1986). This species has also been indicated as a potential vector of *L. infantum* in Iraq and Iran (Sukkar 1985, Azizi et al. 2006). However, while *P. alexandri* collected in field studies have been shown to be infected with *L. infantum* in areas of human disease, the ability of the species to transmit the pathogen to an uninfected host has not yet been demonstrated.

Sand Fly Fever

Sand fly fever is caused by viruses of the Family *Bunyaviridae*, genus *Phlebovirus*. This genus is comprised of at least 37 different serotypes, belonging to 9 distinct species (Tesh 1988, Xu et al. 2007, Charrel et al. 2009). Sand fly transmitted viruses in this genus include: Punta Toro virus, Toscana virus, the sand fly fever Naples and Sicilian viruses (Tesh 1988, Perrone et al. 2007). Punta Toro virus has been isolated from patients in Panama and Colombia (Perrone et al. 2007). Toscana virus is transmitted by *Phlebotomus perniciosus* and is one of the major causes of meningitis in the Mediterranean (Perez-Ruiz et al. 2007, Venturi et al. 2007). The Naples and Sicilian viruses are responsible for Sandfly Fever in the Mediterranean Basin, Northern Africa, and through the Middle East (Tesh 1988).

Leishmaniasis

Leishmaniasis is caused by protozoan parasites in the family Trypanosomatidae (Order Kinetoplastida), genus *Leishmania*. Within the *Leishmania*, there are three subgenera: *Leishmania, Viannia, and Sauroleishmania* (Lainson and Shaw 1987, Correa et al. 2005, Bates 2007, Asato et al. 2009). The subgenus *Sauroleishmania* causes leishmaniasis in reptiles and is transmitted by the sand fly genus *Sergentomyia*. While

the taxonomy and systematics of the *Leishmania* spp. is a developing area, recent molecular work analyzing the cytochrome b generally supported the classical classification introduced by Lainson and Shaw in 1987 (Asato et al. 2009).

Worldwide, 350 million people in 88 countries are at risk of at least one form of leishmaniasis (Desjeux 2004). Annually, there are approximately 1-1.5 million new cases of cutaneous leishmaniasis (CL) and 500,000 new cases of visceral leishmaniasis (VL) (Desjeux 2004). In 2001, there were approximately 59,000 deaths due to leishmaniasis (Campanini and Haden 2002).

There are several forms of leishmaniasis. These include: CL, diffuse cutaneous leishmaniasis (DCL), mucocutaneous leishmaniasis (MCL), and VL. CL appears as a lesion at the site of inoculation with the parasite. Depending on the species of *Leishmania* responsible for the infection, the wound may or may not self-heal (Magill 2000). In DCL infections, lesions appear in various places on the body as a result of a defective cell-mediated immune response (Magill 2000, Desjeux 2004). These lesions do not self-heal and patients often relapse after treatment (Desjeux 2004). MCL appears as lesions in the mucous membranes on the face and can be severely disfiguring (Magill 2000). VL, also known as Kala-Azar or Dum-Dum fever, is a systemic disease characterized by fever, weight loss, hepatosplenomegaly, pancytopenia, and anemia (Magill 2000). When left untreated, VL is generally fatal. Tables 1 and 2 give the *Leishmania* spp. parasites that cause human disease, the form of disease each causes, and vector species.

Leishmaniasis in the Middle East

The different forms of leishmaniasis are often characterized by their ecoepidemiology. In the Middle East, the four major species of concern have different epidemiological patterns. These are: zoonotic cutaneous leishmaniasis (ZCL), anthroponotic cutaneous leishmaniasis (ACL), zoonotic visceral leishmaniasis (ZVL), and anthroponotic visceral leishmaniasis (AVL).

ZCL is caused by *L.major* and is transmitted by *P. papatasi*. Several different species of rodents serve as the reservoirs for this disease across its range (Schlein et al. 1984, Peters et al. 1985, Mehrabani et al. 2007). *Leishmania major* is present throughout the Middle East. ZCL effects human populations when immunologically naïve individuals enter areas with active zoonotic disease transmission, such as through migration, habitat modification from irrigation and agriculture, and movement of non-immune individuals into endemic areas (Seimenis et al. 2006).

ACL is caused by *L. tropica* and is primarily transmitted by *P. sergenti*. In this transmission pattern, the disease circulates solely in the human population. *Leishmania tropica* is endemic in many cities and villages in the Middle East (Magill 1995). ACL can be a major problem in areas with large migrations from rural areas into poor urban environments, where there is civil unrest, and in situations where large numbers of people are displaced due to natural disasters (Desjeux 2001). Kabul, Afghanistan is particularly affected by ACL and documents the highest incidence of CL worldwide (Faulde et al. 2008).

ZVL is caused by *L. infantum* and is suspected to be transmitted by *P. alexandri*, *P. halepensis*, and *P. kanelakii* in the Middle East (Lawyer and Perkins 2000, Azizi et al. 2006). *Leishmania infantum* is responsible for VL across the Mediterranean Basin,

through the Middle East, and into Central Asia. Dogs are thought to be the main reservoir of this disease; however, other animals associated with humans may enter the transmission cycle in the peridomestic environment (Dereure et al. 2003, Maroli et al. 2007, Maresca et al. 2009).

AVL is caused by *L. donovani* and is vectored by a number of species, including *P. alexandri* and *P. argentipes* (Lawyer and Perkins 2000). Leishmania donovani is responsible for VL in the Indian subcontinent, China, Iraq, and portions of Africa. As in the case of ACL, this form of leishmaniasis circulates among the human population without an animal reservoir. Risk factors for epidemics of this disease include migration from non-endemic areas, civil unrest, widespread malnutrition, and disruption in health care (Desjeux 2001).

Leishmania lifecycle

The *Leishmania* parasite undergoes cyclo-propagative development in the sand fly vector. The *Leishmania* have been grouped into three sections based on the position the parasite occupies in the sand fly alimentary tract. These are (Lainson and Shaw 1987, Bates 2007):

1. Section Hypopylaria – *Leishmania* development occurs in the hind gut of the sand fly. Transmission is not well understood. Only occurs in the development of the subgenus *Sauroleishmania* in *Sergentomyia* spp. of sand flies.
2. Section Peripylaria – Prior to migration to the anterior portion of the alimentary tract, the majority of the *Leishmania* promastigotes are located

around the pylorus in the hind gut. This occurs in members of the subgenus *Viannia*.

3. Section Suprapylaria – Parasite development occurs in the midgut prior to movement to the foregut. This occurs in members of the subgenus *Leishmania*.

Aside from the location of parasite development in the sand fly vector, the lifecycle is the same for the subgenera *Leishmania* and *Viannia*.

When feeding upon an infectious host, a sand fly ingests amastigotes. Once in the midgut, the peritrophic membrane develops around the blood meal. Within 12-18 hours of ingestion, amastigotes develop into procyclic promastigotes, which are not very motile and replicate within the peritrophic membrane (Sacks and Kamhawi 2001, Bates 2007). Approximately 18 – 24 hours after ingestion, the procyclic promastigotes begin multiplying rapidly and developing into motile promastigotes that appear in rosette formations (Lainson and Shaw 1987, Sacks and Kamhawi 2001). From 36-60 hours after ingestion, the promastigotes develop into nectomonads (Sacks and Kamhawi 2001). The parasites move to the anterior portion of the midgut, escaping the peritrophic membrane, and attaching to the microvilli of the epithelial cells by approximately 72 hours post ingestion (Sacks and Kamhawi 2001, Bates 2007). Approximately one week after ingestion, the parasites move to the stomodeal valve, which is located at the posterior end of the foregut (Molyneux and Killick-Kendrick 1987, Sacks and Kamhawi 2001). Once at the stomodeal valve, they transform into leptomonad promastigotes, multiply, and produce promastigote secretory gel (PSG) (Rogers et al. 2002, Gossage et al. 2003). Some of the leptomonads develop into haptomonads and attach to the surface of the

valve, while others develop into metacyclic promastigotes (Sacks and Kamhawi 2001, Rogers et al. 2002). A PSG plug develops at the anterior of the midgut, containing leptomonad promastigotes and the infectious metacyclic promastigotes (Bates 2007). When the sand fly attempts to feed, the blockage in the alimentary system created by the PSG plug and the parasites at the stomodeal valve leads to regurgitation and transmission of the metacyclic promastigotes (Rogers et al. 2004).

When the sand fly feeds, the mouthparts cut the skin, creating a subcutaneous pool of blood from which they feed. The metacyclic promastigotes enter the vertebrate host during feeding. In addition to the movement of the parasites, the sand fly injects saliva into the host, triggering an immune response in individuals previously exposed to sand fly bites (Silva et al. 2005). As part of this response, neutrophils, macrophages, and eosiniphils move to the bite wound (Silva et al. 2005). Macrophages are the eventual host cells for *Leishmania* spp.; however, the mechanism by which the parasites are able to invade the macrophage cells without being killed has not been definitively determined (John and Hunter 2008). Most of the promastigotes that enter the host are initially taken up by the neutrophils; however, macrophages will also directly phagocytize the promastigotes when neutrophils have been depleted through apoptosis (Peters et al. 2008). One theory, dubbed the “Trojan horse” model, argues that the infected neutrophils are phagocytized by macrophage cells as they die, allowing the *Leismania* parasite to enter macrophages without being detected (van Zandbergen et al. 2007). Another theory is that the neutrophils undergoing apoptosis release promastigotes that are well adapted to survive in the macrophage (John and Hunter 2008, Peters et al. 2008). Once successfully

inside the macrophage cells, the promastigotes transform into amastigotes and multiply (Lainson and Shaw 1987).

Military Medical Importance

Sand fly fever virus (SFF) infection caused severe morbidity during WWII, with approximately 11,000 cases diagnosed among Allied Forces in North Africa (Konstantinou et al. 2007). In 2007, an outbreak of SFF was identified among service members in central Iraq, with 14 cases confirmed serologically (Ellis et al. 2008).

In World War II there were approximately 75 cases of visceral leishmaniasis (VL) and 1500 cases of cutaneous leishmaniasis (CL) among Allied Forces serving in the Middle East and Mediterranean regions (Most 1968). Leishmaniasis has been an ongoing health risk for members of the Multi-National Force of Observers (MFO) serving in the Sinai desert in Egypt (Fryauff et al. 1993). Thirty cases of leishmaniasis were diagnosed among 700,000 service members deployed in support of Operation Desert Storm from 1990 - 1991 (Martin et al. 1998). During the first two years of Operation Iraq Freedom (OIF), 0.23% of U.S. service members deployed in support of OIF were diagnosed with leishmaniasis (Aronson et al. 2006).

In response to the emerging leishmaniasis problem in OIF in 2003, sand flies collected by U.S. military preventive medicine units in Iraq were shipped to the 520th Theater Area Medical Lab (TAML), which was located in Tallil, Iraq. These flies were then sent on to the Walter Reed Army Institute of Research (WRAIR) in Silver Spring, Maryland. Upon redeployment of the TAML in the fall of 2003, the flies were shipped directly from preventive medicine units to WRAIR. Once at WRAIR, 10% of the flies

were randomly selected for identification, with the remainder being reserved for molecular analysis for *Leishmania* (Coleman et al. 2006). In 2006, the Centers for Health Promotion and Preventive Medicine North (CHPPM-N), located at Ft. Meade, Maryland took over support for the sand fly program. In 2007, the responsibility again shifted to the Centers for Health Promotion and Preventive Medicine – Europe (CHPPM-E). Upon moving under the purview of CHPPM, the program changed slightly, with all female sand flies being pooled for molecular identification of Leishmania parasites (personal communication, Nancy Scott). The male flies are retained in storage at CHPPM-E and are being shipped to WRAIR for eventual identification and cataloging at the Walter Reed Biosystematics Unit (WRBU).

Sand Fly Biology

Lifecycle

The female sand fly oviposits 30 to 70 eggs, either singly or in clusters (Lane 1993). The eggs are deposited in areas that provide moisture, have temperatures between 15.6^{o}C and 26.7^{o}C, are protected from desiccation, and are near a larval food source (Lawyer and Perkins 2000). The sand fly larvae progress through four larval instars, feeding on decaying organic matter. Depending on the species, larvae may burrow through the substrate or feed at the surface (Lane 1993). Upon reaching the fourth instar, environmental extremes cause some species (such as *Phlebotomus ariasi* in the Mediterranean basin) to enter either diapause or quiescence (Lane 1993). The duration of the larval stage varies by species and temperature (Srinivasan and Panicker 1993). In the laboratory, sand fly larvae usually develop in 3-8 weeks (Lawyer and Perkins 2000).

In preparation for pupation, fourth instar larvae stop feeding and move to a drier location (Lawyer and Perkins 2000). The larval skin from the fourth instar is maintained at the posterior end of the pupae and is used to attach to the substrate (Lawyer and Perkins 2000). Pupation lasts approximately one to two weeks. At the end of the pupal stage, adult males emerge 24 to 48 hours before females and reach sexual maturity after 24 hours, when the external genitalia have completed a 180^{o} inversion (Lawyer and Perkins 2000). Adult females emerge sexually mature from the pupal stage.

Behavior

Both male and female adult sand flies require sugar meals. *Phlebotomus papatasi* feed indiscriminatly on all plant tissues either by piercing the tissue with their mouthparts or by feeding directly on the plant surface (Schlein and Warburg 1986). Field experiments in Jordan found an association between plant tissue in the gut of flies and increased longevity, indicating that the sugar meal is key for sand fly survival (Schlein and Jacobson 1999). More recent research demonstrated that *P. papatasi* preferentially feed on flowering bushes over non-flowering bushes (Muller and Schlein 2004).

Female sand flies usually require a blood meal for egg production; however, in the absence of a blood source, some species can produce offspring autogenously (el-Kammah 1973, Srinivasan and Panicker 1993). Blood meal analyses have demonstrated that *P. papatasi* are primarily anthropophilic, but they can also be opportunistic feeders on rodents, bovines, canids, equines, and birds (Mukhopadhyay and Chakravarty 1987, el Sawaf et al. 1989, Srinivasan and Panicker 1992b, Yaghoobi-Ershadi et al. 1995, Bongiorno et al. 2003, Yaghoobi-Ershadi et al. 2005). *Phlebotomus alexandri* is also

primarily anthropophilic; however, they have also been found to feed on rodents and sheep (Guan et al. 1986, Azizi et al. 2006).

Sand flies feed between dusk and dawn. One study in India showed peak *P. papatasi* blood feeding activity occurred at 0100 (Srinivasan and Panicker 1992a). Research in Egypt found that 83% of the female sand flies that enter houses at night do so between 2400 hours and dawn with the majority of entering females taking a fresh blood meal during that time (El Said et al. 1986).

In a capture and release study, blood fed females of Mediterranean species, *Phlebotomus ariasi,* dispersed less than 250 m from their host source in a one week time frame post feeding. This is believed to be the length of time required for the blood meal to be digested and for oogenesis to take place (Killick-Kendrick et al. 1986). After the first week, the blood fed females dispersed up to 1 km from the release point (Killick-Kendrick et al. 1986). The increased travel distance after blood meal digestion is likely due to the need to seek suitable oviposition habitat. Unfed female *P. ariasi* released in the same study dispersed away from the release point more quickly, likely in search of a blood meal; however, the majority remained within approximately 1km of the release point (Killick-Kendrick et al. 1986). Males of the same species remained within 600 m of the release point (Killick-Kendrick et al. 1986).

Sand flies are relatively weak fliers. One study found that the number of sand flies collected using a vehicle mounted net was inversely related to wind speed, with 3.5 m/s estimated to be the highest wind speed at which sand flies can fly (Roberts 1994). Another study in Palestine found that sand flies were most abundant at wind speeds less

than 0.3 m/s (Sawalha et al. 2003). The maximum flight speed for *P. ariasi* was estimated at between 0.65 and 0.70 m/s (Killick-Kendrick et al. 1986).

Few field studies have focused on the reproductive behavior of sand flies. Higher rates of insemination have been demonstrated in female *P. papatasi* collected exiting rodent burrows compared to females collected in open fields (Yuval and Schlein 1986b). This indicates that mating may be occurring in the rodent burrows. Furthermore, analysis of female spermatheca after oviposition indicates that female *P. papatasi* must mate once for each gonotrophic cycle (Yuval and Schlein 1986b). One field study looking at nocturnal activity in sand flies found that the majority of gravid females exit rodent burrows in the first half of the night, from 1800-2400 (Yuval and Schlein 1986a). This may indicate that oviposition, or at least movement to oviposition sites, occurs in the first portion of the night.

Most knowledge of the oviposition behavior of *P. papatasi* comes from studies conducted on laboratory colonies. Even in controlled laboratory colonies, oviposition varies by season, with highest rates of oviposition in the summer and the lowest in the winter (Schlein et al. 1990, Srinivasan and Panicker 1993). The rate of oviposition in laboratory colonies in the winter can be increased by exposing the females to UV light, indicating that seasonal variation in oviposition may be related to UV exposure (Schlein et al. 1990). However, the maintenance of a seasonal difference in oviposition rates in the absence of UV exposure indicates that it is not solely responsible for the observed seasonality. Scratching furrows into the plaster in the ovipot and adding cow manure to the cages have both been shown to increase fecundity in laboratory colonies (Schlein et al. 1990).

There is a dirth of information on natural breeding sites of sand flies due to difficulties in locating immature forms of these insects (Feliciangeli 2004). In general, suitable breeding sites have a relative humidity greater than 80% and are high in organic matter (Sivagnaname and Amalraj 1997). Breeding sites of *P. papatasi* have been identified in rodent burrows, latrines, animal shelters, human dwellings, on the sides of cisterns, and in caves (Sivagnaname and Amalraj 1997, Feliciangeli 2004). Singh et al (2008) found *P. argentipes* and *P. papatasi* in soil samples taken from cattle sheds and human houses in Bihar, India. *Phlebotomus argentipes* was found in greater abundance in the alkaline soil associated with cattle, while *P. papatasi* was more abundant in the pH neutral soil found in human houses (Singh et al. 2008). Documented resting sites include rodent burrows, houses, animal shelters, and accumulations of garbage (Beier et al. 1986, El Said et al. 1986, Yaman and Ozbel 2004).

Distribution and Ecology

Sand flies in the Old World are chiefly distributed in the subtropics; whereas, New World sand flies are mainly in the tropics. They are limited to areas that maintain temperatures above 15.6^{o}C for at least 3 months (Lawyer and Perkins 2000). Species may be found at altitudes ranging from below sea level (Dead Sea area) to more than 2800 m above sea level (Lawyer and Perkins 2000).

Sand fly populations vary in abundance throughout the year. This seasonality is governed by environmental factors, such as temperature and precipitation, and the availability of suitable hosts (Wasserberg et al. 2003a, Boussaa et al. 2005a). Seasonal patterns appear to be species specific; however, research has shown that the patterns of seasonality for the same species (i.e., *P. sergenti*) in different habitats can vary

(Kravchenko et al. 2004). In general, sand flies are most abundant at the end of the rainy season and are least abundant at the end of the dry season (Lawyer and Perkins 2000, Salomon et al. 2002).

Phlebotomus papatasi

Historically, *P. papatasi* is the most collected species of sand fly in Iraq (Al-azawi and Abul-hab 1977, Abul-hab and Ahmed 1984). It is also one of the most abundant species in collections by military entomologists during OIF (Coleman et al. 2007). This species is most abundant in areas with a mean minimum temperature of 16°C and mean maximum temperature of 44°C from May to October (Cross et al. 1996). Research conducted in the Sinai Desert, Egypt found that *P. papatasi* was present when the night time temperature ranged from 15 – 30^{o}C (Hanafi et al. 2007).

Table 3 summarizes studies that have examined seasonality of *P. papatasi* at various locations across its range. An examination on the affect of altitude on population levels of *P. papatasi* in Turkey found this species at elevations ranging from near sea level to over 1100 meters above sea level (Belen et al. 2004). While *P. papatasi* occupies a large range in the Old World and displays some morphological differences across its range, it does not appear to display significant genetic variation across populations (Esseghir et al. 1997, Kassem et al. 1999, Belen et al. 2004).

Phlebotomus alexandri

Phlebotomus alexandri has a wide range in the Old World, with records as far east as Spain and Morocco and west to China and Mongolia (Depaquit 1997). This

species has historically been collected in large numbers throughout the Mesopotamian basin in Iraq (Abul-hab and Ahmed 1984). Along with *P. papatasi*, *P. alexandri* has consistently been one of the two most abundant species collected during OIF (Coleman et al. 2007). While this species is not as well researched as *P. papatasi*, a few studies have given insight into its range and ecology.

In the Sinai, this species has been recorded at altitudes ranging from 550m above sea level to 1500 m above sea level (Kamal et al. 2003). A survey of sand flies in Djibouti found that *P. alexandri* was the most widely distributed peridomestic species and was found in all habitats except mountain forests (Fryauff et al. 1995). A survey in Oman using a vehicle mounted collection net found *P. alexandri* to be the most abundant species of *Phlebotomus*, with collection number increasing with low humidity, low wind speed, and high temperature (Roberts 1994). Studies in Turkey, Iraq, and Iran have found that *P. alexandri* populations have a seasonal peak from August – September (Javadian and Nadim 1975, Sukkar 1985, Sukkar et al. 1985, Toprak and Ozer 2005). At Tallil Air Base, Iraq, *P. alexandri* was more abundant than *P. papatasi* during the months of April and May, but less abundant in August and September (Coleman et al 2007).

The wide range of this species and reported morphologic variation across the range has raised the question of whether this is a single species or a species complex (Depaquit 1997, Depaquit et al. 2000, Kakarsulemankhel 2004). Variation has been noted in the structure of the female spermatheca, female genital armature, and the general size and coloration of this species across its range (Depaquit 1997, Kakarsulemankhel 2004) However, no work has been conducted to date on this issue. If *P. alexandri* is actually a species complex, members of this complex may have different distributions,

ecology, and vector potential. Determining the status of this species could help better define its medical importance and increase understanding of its ecology.

Genetic Diversity and Vector Competence

In order for an arthropod to be confirmed as a disease vector, a deliberate process of vector incrimination must take place. The steps in vector incrimination are as follows (Eldridge 2000):

1. Collection of naturally infected arthropods in the field.
2. Spatial and temporal association between the occurrence of the disease in the human population and the presence of the suspected vector.
3. The suspected vector must make contact with the host (i.e., feeding) that is sufficient to transmit the pathogen in nature.
4. The suspected vector must become infective and transmit the pathogen in the laboratory environment.

The vector competence of a species is a measure of how susceptible the vector is to infection with a pathogen and how efficiently that vector can transmit the pathogen to novel hosts (Eldridge 2000). Vector competence can vary among populations of a given vector species and among species within a species complex, having implications on disease epidemiology.

An analysis of the vectorial efficiency of six strains of *Aedes polynensiensis* for *Wuchereria bancrofti* showed differences among the strains (Failloux et al. 1995). *Aedes aegypti* populations in Australia have been found to vary in vector competence for dengue type 2 and 4 viruses between two populations (Knox et al. 2003). Members of the *Anopheles maculipennis* and *An. gambiae* species complexes vary in their ability to transmit malaria as a result of behavioral and ecological differences between species within the complexes (Tabachnick and Black 1995, Proft et al. 1999, Bogh et al. 2003).

Intraspecific genetic variation can lead to variation in vector competence and disease ecology (Tabachnick and Black 1995, Black and Munstermann 1996). Therefore, identifying genetic variation among vector populations is important in understanding the potential role vectors play in disease transmission. A good deal of research has been done into the question of whether or not the New World sand fly species, *Lutzomyia longipalpis* s.l., is a species complex (Bauzer et al. 2007). While it appears that controversy remains regarding that question, molecular research conducted on *Lu. longipalpis* s.l. populations has revealed that there are differences in the expression of maxadilan, a salivary peptide and vasodilator present in *Lutzomyia* spp. sand flies (Mukhopadhyay et al. 1998, Lanzaro et al. 1999). A growing body of evidence is linking the level of expression of maxadilan to the development of leishmaniasis; therefore, any variation between populations of *Lu. longipalpis* s.l. may impact vector competence and disease expression (Lainson and Rangel 2005).

Previous work on the molecular systematics of the subgenus *Paraphlebotomus* has used the internal transcribed spacer 2 (ITS2) of ribosomal DNA (rDNA) to distinguish between species (Depaquit et al. 2000). Genetic variations among *P.(Paraphlebotomus) sergenti* populations in Iran have been identified, this may have implications for the epidemiology of *L. tropica* (Moin-Varizi et al. 2007). In addition, mitochondrial cytochrome b has been used to examine genetic differences within a variety of *Phlebotomus* and *Lutzomyia* species (Esseghir et al. 1997, Ready et al. 1997, Parvizi et al. 2003, Pesson et al. 2004).

Niche Modeling

In recent years, the use of remotely sensed data has gained in popularity in many areas of biology, including epidemiology (Peterson 2006). Along with this, a number of modeling approaches have been developed to answer basic questions about the distribution, ecology, and potential range of a species in the absence of comprehensive sampling data. These techniques are commonly referred to as ecological niche modeling (ENM) or species distribution modeling (SDM).

Joseph Grinell originally presented the idea that species inhabit specific areas (the niche) that can be defined by the species' biological requirements for survival (Grinnell 1917). The ecological niche is determined by four interacting factors, these are (Costa et al. 2002, Soberon and Peterson 2005, Peterson 2008):

1. Biotic factors – Interactions with other species including: predators, pathogens, prey, and parasites.
2. Abiotic factors – The environmental factors that determine whether or not a species can survive in a given location (climate, soil, etc.).
3. The ability of a species to disperse to new areas. This includes any physical barriers (such as mountain ranges, bodies of water, and deserts) to dispersion and well as the biological capacity or propensity of a species to disperse.
4. The ability of the species to adapt to new environments.

Together, these factors determine the actual and potential geographic distribution of a species (Soberon and Peterson 2005). ENM techniques suggest new hypotheses and can be used to suppor theoriesbased on the knowledge of these factors for a given species.

Niche modeling has been increasingly applied to disease ecology. Models of monthly predictions of dengue fever in Mexico have been created based on mosquito activity (Peterson et al. 2005). Niche modeling has been used to help prevent anthrax in wildlife and livestock, by predicting the expected distribution of the pathogen *Bacillus anthracis* in the environment (Blackburn et al. 2007). Niche models of malaria vectors in the *Anopheles gambiae* complex have been developed for undersampled regions of Africa (Levine et al. 2004). The distributions of *Triatoma* spp. vectors of Chagas' disease in the Americas have been examined in an effort to better refine vector control programs (Costa et al. 2002, Peterson et al. 2002). A number of studies have focused on both present and potential distributions of *Lutzomyia* spp. vectors of cutaneous and visceral leishmaniasis in Central and South America (Peterson and Shaw 2003, Peterson et al. 2004, Nieto et al. 2006, da Costa et al. 2007).

Study Area

The work in this dissertation will focus on sand flies in the Middle Eastern region. Definitions of the actual extent of the Middle East vary. For the purposes of this study, we are including portions or all of the following countries: Afghanistan, Armenia, Azerbaijan, Cyprus, Egypt, India, Iran, Iraq, Israel, Jordan, Kuwait, Kyrgyzstan, Lebanon, Oman, Pakistan, Qatar, Saudi Arabia, Syria, Tajikistan, Turkey, Turkmenistan, the United Arab Emirates, Uzbekistan, and Yemen (Figure 1). Portions of this research focus on Iraq, using collections performed by military entomologists during Operation Iraqi Freedom. Figure 2 contains a map of Iraq and general sand fly sampling locations.

There is wide variation across different portions of the Middle East. Across the region, the mean daily temperatures in January can range from -10°C to 25°C and precipitation ranges from 0 mm to 200 mm (Pilifosova 2000). In July, the mean daily temperatures across the region range from 20°C to more than 35°C and precipitation can range from 0 mm to 500 mm (Pilifosova 2000).

Strong winds and dust storms can be a problem in some portions of the Middle East. The strongest storms occur across Iran, Iraq, Syria, Kuwait, Bahrain, Qatar, the United Arab Emirates, the southern portion of Saudi Arabia, Saudi Arabia, Yemen, and Oman (Kuteil and Furman 2003). In these areas, blowing dust is most problematic in the springs through fall. Visibility is reduced by airborne dust more than 30% of the time in the summer and approximately 5% of the time in the winter (Kuteil and Furman 2003).

Research Goals

The goal of this research is to better understand the biology and ecology of sand fly vectors in the Middle East, focusing on *P. papatasi* and *P. alexandri*, the two most abundant species in collections by military entomologists during Operation Iraqi Freedom (OIF). This research examines the following hypotheses:

1. The nocturnal activity of sand flies in Iraq is influenced by climatological variables

2. The geographic distribution of *P. papatasi* and *P. alexandri* in Iraq is associated with vegetation.

3. Environmental variables serve as predictors of *P. papatasi* and *P. alexandri* presence in a given area

4. There is genetic variation across the range of *P. alexandri*.

The first two hypotheses are tested by focusing on sand flies in Iraq, using collection records from OIF. The first hypothesis is examined through multivariate analysis of weather data and sand fly collection data at Tallil Air Base, Iraq. The second hypothesis is explored using geographic information systems (GIS) to examine the relationship between the normalized difference vegetation index (NDVI) and the presence of the sand fly species in Iraq. Niche modeling is used for testing the third hypothesis to examine the ecology of *P. papatasi* and *P. alexandri* in the Middle East and to develop models predicting the occurrence of these species in that region. The fourth hypothesis looks more closely at *P. alexandri* in an effort to determine the level of genetic variation across its range. As genetic variation may ultimately impact the vector competence of this species, this is an important step in understanding the medical importance of this species.

Table 1. *Leishmania* (*Viannia*) spp. parasites, the disease they cause, and vectors (Lainson and Shaw 1987, Lawyer and Perkins 2000, Bates 2007, Asato et al. 2009)

Complex	*Leishmania* spp.	Region[1]	Form[2]	Vector(s)
L. braziliensis	*L.(V.) braziliensis*	NW	CL, MCL	*Lu. Carrerai* *Lu. ovallesi* *Lu. wellcomei* *Lu. Whitmani*
	L. (V.) colombiensis	NW	CL	*Lu. hartmanni** *Lu. gomezi** *Lu. panamensis**
	L. (V.) guyanensis	NW	CL	*Lu. anduzei* *Lu. Umbratilis*
	L. (V.) lainsoni	NW	CL, MCL	*Lu. ubiqutalis**
	L. (V.) naiffi		CL	*Lu. squamiventris**
	L. (V.) panamensis	NW	CL	*Lu. gomezi** *Lu. panamensis** *Lu. Trapidoi*
	L. (V.) peruviana	NW	CL	*Lu. peruensis** *Lu. verrucarum**
	L. (V.) shawi	NW	CL	*Lu. whitmani**

* Suspected vector.

[1] NW (New World); OW (Old World)

[2] CL (Cutaneous leishmaniasis); MCL (Mucocutaneous leishmaniasis); VL (Visceral leishmaniasis)

Table 2. *Leishmania* (*Leishmania*) spp. parasites, the disease they cause, and vectors (Lainson and Shaw 1987, Lawyer and Perkins 2000, Bates 2007, Asato et al. 2009)

Complex	*Leishmania* spp.	Region[1]	Form[2]	Vector(s)
L donovani	*L. (L.) chagasi*	NW	VL	*Lu. evansi** *Lu. Longipalpis*
	L. (L.) donovani	OW	VL	*P. alexandri* *P. argentipes* *P. celiae* *P. martini* *P. mongolensis* *P. orientalis*
	L. (L.) infantum	OW	CL, VL	*P. alexandri** *P. ariasi* *P. perfiliewi* *P. perniciosus* *P. sichuanensis* *P. smirnovi*
L. Mexicana	*L. (L.) amazonensis*	NW	CL, MCL	*Lu. flaviscutellata* *Lu. olmeca nociva*
	L. (L.) garnhami	NW	CL	*Lu. youngi**
	L. (L.) mexicana	NW	CL	*Lu. anthophora* *Lu. diabolica* *Lu. olmeca olmeca**
	L. (L.) pifanoi	NW	CL	*Lu. flaviscutella*
	L. (L.) venezuelensis	NW	CL	*Lu. olmeca**
L. tropica	*L. (L.) aethiopica*	OW	CL	*P. longipes* *P. pedifer*
	L. (L.) killicki	OW	CL	*P. alexandri** *P. chaubaudi** *P. papatasi**
	L. (L.) major	OW	CL	*P. caucascicus** *P. duboscqi* *P. papatasi* *P. saheli**
	L. (L.) tropica	OW	CL, VL	*P. aculeatus** *P. guggisbergi* *P. sergenti*

* Suspected vector.
[1] NW (New World); OW (Old World)
[2] CL (Cutaneous leishmaniasis); MCL (Mucocutaneous leishmaniasis); VL (Visceral leishmaniasis)

Table 3. Studies examining seasonal patterns of *Phlebotomus papatasi*.

Location (cite)	**Timeframe**	**Season**	**Peak**
El Agamy, Egypt (Beier et al. 1986)	1983 – 1984	April – December	June - July
Sinai, Egypt (Hanafi et al. 2007)	1989 – 1991	April – November	May July
India (Srinivasan et al. 1993)	Mar 1988 – Feb 1990	Year-round	September – October
Iran (Yaghoobi-Ershadi and Javadian 1997)	Mar 1991 – Mar 1992	May – Nov	June September
Shiraz City, Iran (Reza and Mansour 2006)	Jan – Dec 2004	May – Nov	September
Arava, Isreal (Wasserberg et al. 2003b)	Apr – Oct 1999	Apr – Oct	April October
Negev, Israel (Wasserberg et al. 2003b)	Apr – Oct 1999	Apr – Oct	April-May
Morocco (Boussaa et al. 2005b)	Oct 2002 – Sep 2003	Year-round	November May
Saudi Arabia (Morsy et al. 1995)	Jan – Dec 1994	Mar – Dec	June September

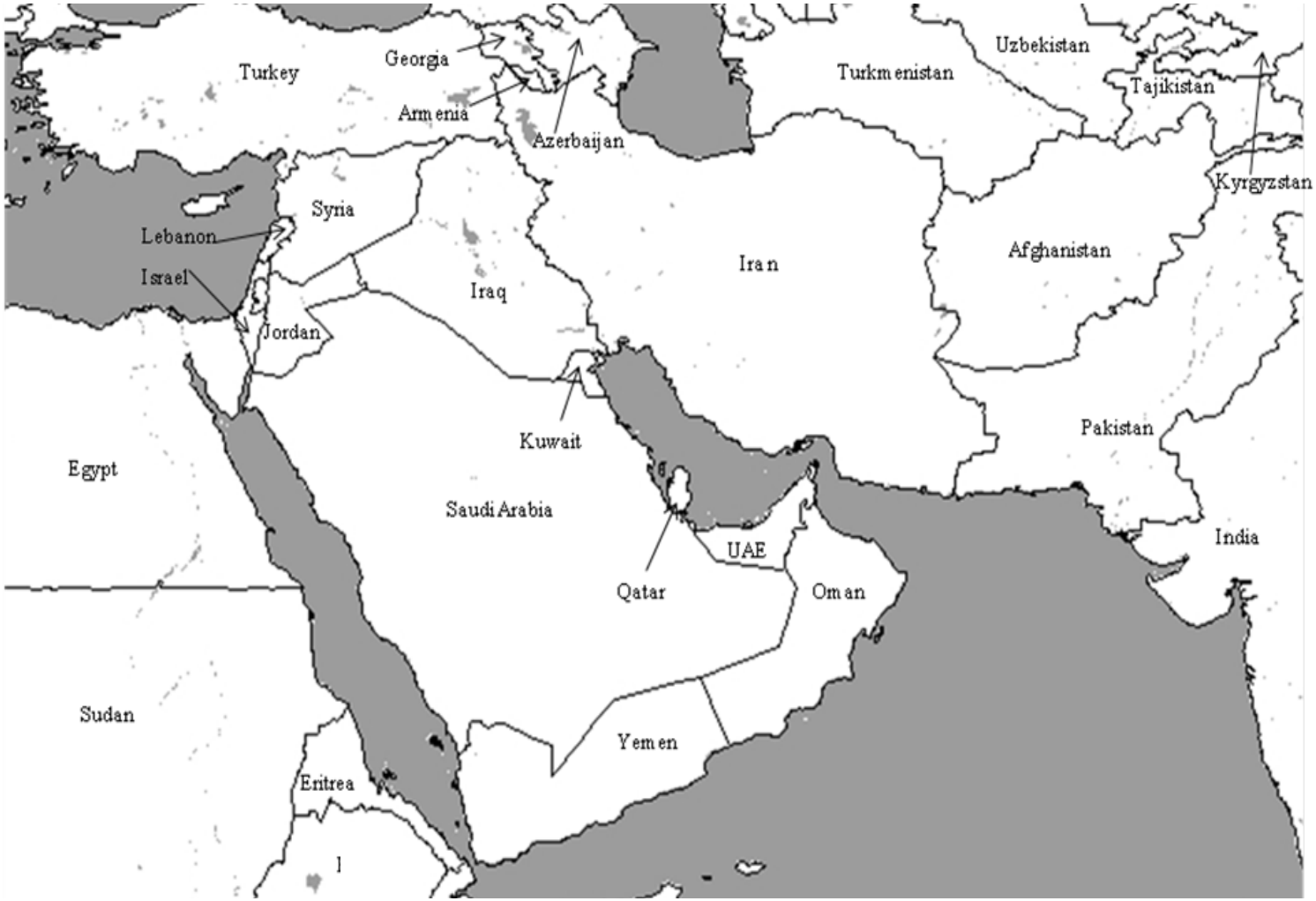

Figure 1. Map of the region included in portions of this research.

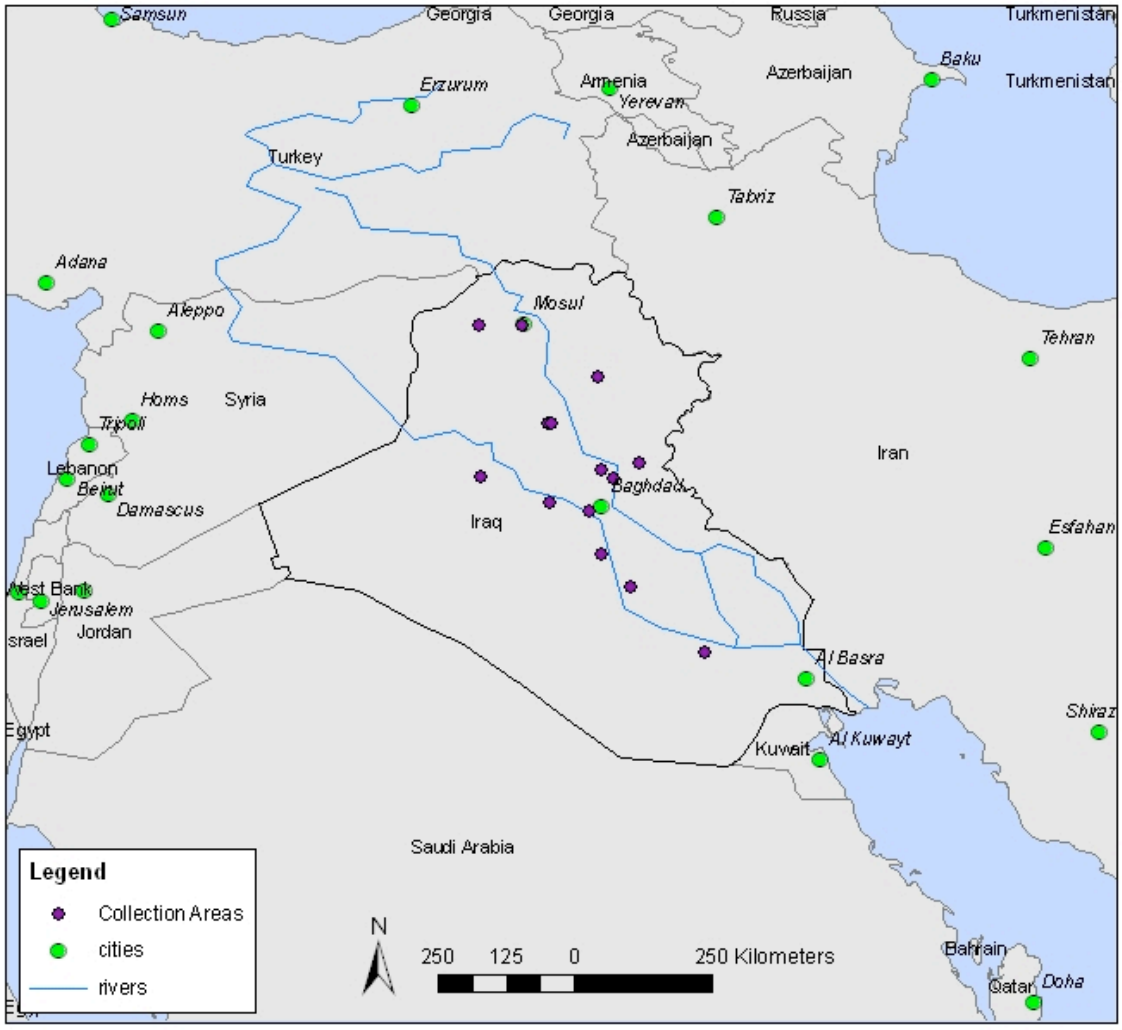

Figure 2. Map of Iraq and surrounding areas. Locations marked with purple circles are general locations where sand flies were collected from 2003-2005.

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Chapter 2

The role of environmental conditions on sand fly activity

Abstract

The present study examines the effect of environmental factors on sand fly activity, as measured by light trap collections. Traps were placed from April – October 2003 and 2004 in Tallil, Iraq. The meteorological conditions during this period are described. Wind speed, temperature, dew point, percent sky cover, and moon illumination were entered into principal components analysis (PCA). The resulting principal components (PCs) were entered into stepwise regression in order to develop a model of the impact of the weather on sand fly collections. Moon illumination had a strong inverse relationship with collection numbers, indicating that increased moon illumination could decrease the efficacy of the standard CDC light trap. Wind speed and percent sky cover also had inverse relationships to sand fly collections while temperature displayed a direct relationship to sand fly collections. Based on a review of the entire dataset, we conclude that sand fly activity in Tallil, Iraq is highest on warm, clear nights, with low wind speed.

Keywords: sand fly, *Phlebotomus*, ecology, light trap, Iraq

In March 2003, U.S. military forces established operations at Tallil Air Base (TAB) in south-central Iraq. Once in place, military preventive medicine personnel established a Leishmaniasis Control Program (LCP) which focused on risk communication, vector surveillance and control, and personal protective measures (Coleman et al. 2006). As part of the LCP, standard CDC light traps were used to perform sand fly collections from March – October 2003 and April – October 2004. From this work, the predominant vector species present at TAB were identified as *Phlebotomus alexandri* Sinton and *P. papatasi* Scopoli (Coleman et al. 2007). *Phlebotomus alexandri* is a suspected vector of *Leishmania infantum* Nicolle and a proven vector of *L. donovani* Laveran and Mesnil (Sukkar 1985, Guan et al. 1986, Azizi et al. 2006). *Phlebotomus papatasi* is a vector of *L. major* Yakimoff and Schokher (Killick-Kendrick et al. 1985a, Sukkar 1985, Azizi et al. 2006).

While sand flies are present throughout Iraq and there is a long-standing history of both cutaneous and visceral leishmaniasis in this country, relatively little is known about the ecology and behavior of sand flies in Iraq. Studies of *P. papatasi* in the Baghdad area showed that the population levels are highest for that species in August through October (Abul-hab and Mahdi 1970, Abul-hab and Al-Baghdadi 1972), while Coleman et al. (2007) found that *P. alexandri* is more abundant in TAB from April through May, with *P. papatasi* becoming more abundant from August through September. In April and October, sand flies at TAB are more active from 2000 - 2400; however, from May through September, they are more active from 2200-0400 (Coleman et al. 2007). The purpose of the present study was to increase our understanding of the

sand fly behavior and ecology in Iraq by examining the relationship between nocturnal sand fly activity at TAB and meteorological variables.

MATERIALS AND METHODS

Collection of Sand flies. Sand flies were collected in standard CDC style light traps (Model 512, John W. Hock Company, Gainesville, FL) from April through October 2003 and April through October 2004. Traps were placed at 1800 local time and the contents collected by 0800 the next day. The location of each trap was recorded using a hand-held Geographic Positioning System (GPS) device. All sand flies were stored in a -70^{o}C freezer in the field laboratory. For the present study, the number of sand flies collected on a given night were divided by the number of traps set out on that night. This resulted in the number of sand flies per trap night and this value was used as a surrogate measure for sand fly activity in the study. Once the sand flies were sorted, 10-15% of the female flies and 95% of the male flies were packaged and shipped to the Walter Reed Army Institute of Research (WRAIR) in Silver Spring, MD. Once at WRAIR, randomly selected male and female sand flies were slide mounted and identified to species.

Collection of Weather Data: Weather data was provided by the U.S. Air Force Weather Detachment (Station KQXJ) located at Tallil Air Base, Iraq (30^{o}57'N, 046^{o}05'E). Collection of weather data was initiated by the Weather Detachment at 2155 on 22 March and continues to the present. Weather data was collected hourly and daily weather observations recorded on Air Force Form 3803 (Surface Weather Observations). Parameters recorded included the following: (1) local time, (2) universal time, (3) precipitation (in inches), (4) wind direction (true degrees), (5) wind speed (knots), (6) temperature (degrees centigrade), (7) dew point (degrees centigrade), (8) barometric

pressure (inches), and (9) percent of the sky covered. Data from all Air Force Form 3803 for the entire study period was migrated into a master EXCEL file. All data was double-checked prior to initiating analyses. Hourly weather data were condensed into daily records, with the mean, minimum, maximum, and standard error of the mean values calculated for each weather variable.

Statistical Analysis: Only the weather data for days in which traps were placed are used in the statistical analysis to examine the relationship between sand flies per trap night and weather. Principal components analysis (PCA) was used to reduce the large number of independent variables to principal components (PC). The independent variables entering the PCA were: mean, minimum, and maximum wind speed; mean, minimum, and maximum temperature; mean, minimum, and maximum dew point; mean, minimum, and maximum sky cover; and moon illumination. All PCs were then entered into stepwise regression with an alpha of 0.05 to enter and an alpha of 0.05 to remove. The dependent variable was set as the number of sand flies per trap night (Jolliffe 2002). All PCs identified as significant in the stepwise regression were further examined to identify those original independent variables that affect the number of sand flies collected. This was accomplished by multiplying the loading values for each PC in the regression model by the corresponding regression coefficient. The standardized values for each independent variable were then summed (Zuur et al. 2007). This serves to standardize the independent variables and allow for direct comparison among the original independent variables in the regression model created using PCA (Zuur et al. 2007).

RESULTS

General Meteorological Conditions at TAB.

Collection of meteorological data at TAB was initiated by the U.S. Air Force Weather Detachment on 23 April 2003 and has continued to the present. The overall weather conditions at TAB were typical of a semi-arid desert, to include extremely high temperatures, low humidity and blowing wind. A summary of the seasonal weather parameters (wind direction, wind speed, temperature, dew point, precipitation and cloud cover) over the course of the study is provided in Figure 1, while monthly data is provided in Table 1.

Seasonal Conditions: The maximum temperature recorded during the study was 52^{o}C (125.6^{o}F) recorded on 8 August, 2003. The lowest temperature recorded was 0^{o}C, recorded on 24 February, 2004. Maximum daily temperatures $\geq$40^{o}C were recorded on 283 (51.1%) of the 554 days in this study. Temperatures $\geq$40^{o}C occurred between 25 April and 15 October in 2003 and 3 May and 21 October in 2004. Minimum daily temperatures <10^{o}C were recorded on 102 (18.4%) of the 554 days that weather was recorded. Temperatures <10^{o}C occurred between 31 October, 2003 and 8 April 2004. There was an inverse relationship between the dew point and temperatures, with dew points highest in the cool season and lowest in the hot season (Table 1).

The wind at TAB generally blew from the west, with an average daily wind direction (ADWD) of 256 degrees (STD DEV = 103 degrees). An ADWD of 225-315 degrees occurred on 286 (51.6%) of 554 days and 180-360 degrees on 439 (79.2%) of

554 days. The wind occasionally blew from the east, with an ADWD of 45-135 degrees occurring on 40 (7.2%) days and between 0-180 degrees on 115 (20.8%) days. In general, there were no significant differences in the ADWD that occurred during the hot and cool seasons. The maximum wind speed recorded during the study period was 30.38 m/s on 23 April 2003. The average daily wind speed was 4.01 m/s (STD DEV = 2.63 m/s), with the highest winds occurred during June, July and August (Figure 1).

Measurable precipitation occurred on only 26 (4.7%) of 554 days, with a total of 18.9 cm of precipitation occurring during the entire period. The bulk of the rainfall occurred on 6 days (3 and 5 December 2003, and 7, 12 and 23 January and 24 April 2004) when 14.9 cm (78.8% of the total) occurred. No measurable precipitation occurred during the hot season in either 2003 or 2004 (Table 1). The lack of rainfall during the hot season was associated with generally clear skies. The portion of the sky that was covered by clouds was generally <10% in the hot season whereas it was generally >30% in the cool season and during the transition months of April and October (Figure 1; Table 1).

Daily Conditions during the Hot Season: Since sand flies were predominantly active during the hot season, we more closely examined meteorological conditions that occurred during this period. The daily variation in six meteorological parameters during the hot season (May thru September) is presented in Figure 2. Wind direction did not vary significantly over the course of the day; however, wind speed was low in the morning and increased over the course of the day until 1600 hrs, when it normally began to decrease. The high winds in the afternoon were often accompanied by a great deal of blowing dust and debris (sandstorms), as evidenced by the increase in percentage of sky covered during the course of the day (Figure 2). Temperatures during the summer

increased gradually over the course of the day, reaching a peak at around 1300 hrs. Temperatures remained high until around 1900 hrs when they typically began decreasing. Dew points were lowest during the day and gradually increased from sunset until around 0800 hrs. Barometric pressure (ALSTG) did not vary greatly over the course of the average day during the hot season.

Collection of Sand flies.

A total of 78,289 sand flies were collected in 1,455 trap nights at 81 different trap locations during the course of the study. In 2003, traps were set on 97 different nights. The mean number of sand flies per trap night was 49.65, with a minimum of 0.36 sand flies per trap night occurring on 31 October 2003 and a maximum of 244.00 occurring on 1 July 2003. In 2004, traps were set on 27 nights. The mean number of sand flies per trap night was 59.64, with a minimum of 3.08 on 25 October 2004 and a maximum of 298.31 sand flies per trap night on 24 July 2004.

Statistical Analysis. The PCA yielded 13 PCs which are not correlated with one another. The first four PCs had eigenvalues greater than 1 and accounted for 82.1 percent of the variance (Table 2). All thirteen PCs were entered into the stepwise regression procedure. Principle components 4, 5, and 6 were retained in the model ($p<0.05$). The regression model is:

$$\text{Sand flies per trap night} = 51.00 + 12.8PC4 + 24.7PC5 - 16.2PC6$$

The loading values for PC4, PC5, and PC6 were multiplied by the corresponding regression coefficient. The resulting values were then summed across the three PCs for each of the original independent variables (Zuur et al. 2007). The original loading, "standardized" loading, and sums for each original independent variable are in Table 3.

Moon illumination, minimum wind speed, and mean wind speed had the greatest affect on the dependent variable, with all three being inversely related to numbers of sand fly per trap night. Mean, minimum, and maximum temperature and maximum wind speed had moderate positive relationships with the dependent variable. Mean and minimum percent sky cover also had moderate inverse relationships with sand flies collected. The dew point variables and the maximum percent sky cover had little influence on the model.

In order to better understand the unexpected relationship of maximum wind speed to sand fly collections, the weather data was separated out into two periods: day (0600-1800), when sand flies were not being collected, and night (1800-0600), when traps were in place. The analysis was then run with 25 variables: moon illumination plus day and night wind speed, temperature, dew point, and per cent sky cover variables. The end result of the analysis mirrored the results using the 24-hour variables, with the exception of the mean, minimum, and maximum wind speed. The night time wind speed variables all had an inverse relationship with trap collections. The daytime wind speed variables had a direct relationship with trap collections at night. Daytime wind speed and temperature are strongly correlated, with both increasing gradually throughout the daytime hours (Figure 2).

DISCUSSION

The sand fly species collected at Tallil Air base in 2003 and 2004 were *P. alexandri* (30%), *P. papatasi* (24%), *P. sergenti* (1%) and *Sergentomyia* spp. (Coleman et al. 2007). Since the measure used in this study was based on total sand flies, there is no way to determine whether individual species might have had slightly different responses to the environmental variables.

Moon illumination had the strongest influence on the number of sand flies collected in the present study, with increased illumination leading to decreased numbers collected. One study looking at the distance at which sand flies pick up the visual cue from light traps found that *P. ariasi* is attracted to light traps within 2 meters of the trap (Killick-Kendrick et al. 1985b). With increased moon illumination, there is increased ambient light which could create competing light sources, thereby reducing the number of sand flies that would pick up the visual cue from the light trap and move toward it. One concern by some researchers in using light traps is that they can be biased toward more phototropic species (Alexander 2000). A 2003 study undertaken at Tallil compared the standard CDC down-draft trap, the Down-draft trap with a UV bulb, an up-draft CDC trap, and sticky paper augmented with a green chemlight. The standard CDC down-draft trap collected more than the sticky paper and less than the two other light traps (Burkett et al. 2007). In Saudi Arabia, collection numbers of *Phlebotomus* sand flies were higher in light traps compared to sticky traps, with the reverse being true for *Sergentomyia* spp. (Al-Zahrani et al. 1997). Toprak and Ozer (2007) found light traps to be more efficient than either aspirators or sticky traps. Light traps were also found to be more efficient

than aspirator collections of *P. argentipes* in India (Dinesh et al. 2008). Studies comparing the efficacy of light traps to biting collections in Egypt found that the methods are comparable (Fryauff and Modi 1991). While light traps fare well in comparison to other sand fly collection methods, the results of the present study indicate that consideration should be given to supplementing the light trap with a CO^2 source to provide a non-visual attractant cue in cases where there is increased moon illumination and ambient light.

High mean, minimum, and maximum wind speed at night led to decreased numbers of sand flies collected. The number of sand flies collected using a vehicle mounted net in Oman was inversely related to wind velocity, with 3.5m/s thought to be the highest velocity at which sand flies can fly (Roberts 1994). Work in Palestine found that sand flies were most abundant when wind speeds were below 0.3 m/s (Sawalha et al. 2003). The highest mean wind speed in the present study was 6.17 m/s on 12 August 2003, with 4.92 sand flies per trap night. The largest collection occurred on 24 July 2004, with 298.31 sand flies per trap night and a mean wind speed of 1.63 m/s.

The positive relationship between wind speed during the day and night time sand fly activity is a reflection of the correlation of wind speed and other variables. As illustrated in Figure 1, wind speed increases during the day with temperature and sky cover before dropping off in the evening hours. Since highest wind speeds tend to occur during time periods when sand flies are not active, these winds do not directly impact sand fly activity.

In the present study, temperature has a moderately strong direct relationship with sand fly activity. This association is in agreement with other studies that have

demonstrated similar relationships in other sand fly species from other locations. In Morocco, sand flies (primarily *P. papatasi* and *Sergentomyia* spp.) are active year-round, but the density of sand flies collected is positively associated with temperature (Boussaa et al. 2005). Studies in the Middle East, have shown that *P. papatasi* activity starts in the spring, reaches a peak in the late summer, and stops entirely by the end of November (Merdan et al. 1992, Morsy et al. 1993, Janini et al. 1995, Morsy et al. 1995, Yaghoobi-Ershadi and Javadian 1997). In Palestine, sand fly activity is highest when the temperature is in the 24-26^{o}C range (Sawalha et al. 2003). In the present study, sand fly activity is highest from late June through August, when the mean night time temperature is greater than 30ºC. Activity is lowest when the mean night time temperature is 24ºC or less.

Percent sky cover had a moderate inverse relationship with the number of sand flies collected per trap night. Sky cover increased gradually through the day time hours, dropping off at night (Figure 1). The relationship between sky cover and sand fly activity could be related to increased levels of particulate matter in the air due to dust storms that can develop as temperature and wind speed increase late in the day. Such increased particulate matter would likely decrease the ability of the sand flies to fly. Increased particulate matter in the air could also decrease the efficacy of the light traps by causing the light to be more diffuse.

Due to large gaps between sampling days (primarily in 2004) and a lack of weather data prior to 22 March 2003, we were unable to perform a time series analysis to determine what environmental factors might influence sand fly activity weeks or months in the future. One study in India found that soil temperature and moisture one month

before sand fly collection was positively associated with the numbers of sand flies collected. In the future, it would be beneficial to examine the time relationships between environmental variables and sand fly activity in the Middle East. These relationships could give valuable insight to help target and develop control strategies.

Our findings indicate that sand flies in Tallil, Iraq are most active on warm, clear nights with little wind. These results may be used to fine tune sampling strategies and better target control strategies (i.e. area-wide ULV spraying) to days when sand flies will be more active. Further, the findings here indicate that, when activity is measured by means of a light trap, moon illumination can strongly impact the outcome suggesting that adding an attractant to the trap may provide more accurate surveillance data.

Table 1. Average monthly meteorological data from Tallil Air Base (April 2003 until October 2004).

	Average monthly values (Std Dev; Range)					
Month	Total Rainfall (cm)	Mean Temperature (^{o}C)	Mean Dew Point (^{o}C)	Mean Wind Speed (m/s)	Mean Wind Direction (^{o}true)	Mean Sky Cover (%)
Jan	0.2 (0.7; 0-2.9)	13.0 (3.7; 3-21)	8.5 (3.2; 0-17)	3.0 (2.2; 0-14.4)	222.6 (102; 0-360)	46.0 (40.4; 0-100)
Feb	0.0 (0)	14.2 (5.4; 0-28)	3.0 (4.4; -9-15)	3.6 (2.6; 0-13.4)	236.1 (100; 0-360)	29.8 (38.0; 0-100)
Mar	0.0 (0)	20.8 (6.9; 5-37)	0.8 (4.7; -12-12)	3.9 (2.4; 0-13.9)	250.0 (107; 0-360)	36.7 (34.3; 0-100)
Apr	0.2 (0.9; 0-5.7)	24.2 (6.5; 3-40)	2.5 (7.1; -13-19)	4.1 (2.9; 0-18.5)	212.3 (109; 0-360)	40.4 (37.4; 0-100)
May	0.0 (0)	31.7 (6.3; 15-45)	2.0 (4.3; -9-18)	3.7 (2.4; 0-15.4)	231.9 (120; 0-360)	27.4 (33.2; 0-100)
Jun	0.0 (0)	35.7 (6.6; 20-49)	1.6 (3.0; -11-13)	4.8 (2.7; 0.5-14.9)	281.7 (98; 0-360)	7.6 (20.5; 0-100)
Jul	0.0 (0)	37.1 (7.1; 23-50)	2.1 (2.6; -7-10)	5.1 (3.0; 0-16.5)	294.2 (72; 0-360)	6.6 (16.5; 0-87.5)
Aug	0.0 (0)	36.9 (7.2; 23-52)	3.9 (3.2; -6-19)	4.7 (2.7; 0-13.9)	285.0 (84; 0-360)	3.8 (12.6; 0-100)
Sep	0.0 (0)	32.7 (7.5; 17-49)	2.0 (3.1; -6-12)	4.1 (2.6; 0-16.0)	275.6 (91; 0-360)	3.2 (11.2; 0-100)
Oct	<0.1 (0; 0-0.1)	28.2 (6.8; 10-43)	3.8 (4.8; -7-23)	3.0 (2.1; 0-13.9)	219.5 (112; 0-360)	20.1 (28.6; 0-100)
Nov	<0.1 (0; 0-1.0)	17.9 (6.6; 3-33)	3.4 (6.9; -9-19)	3.7 (2.1; 0-13.9)	255.8 (99; 0-360)	39.7 (39.7; 0-100)
Dec	0.12 (0.4; 0-1.7)	12.3 (4.6; 1-27)	7.0 (3.7; -2-15)	3.6 (2.1; 0-18.5)	250.7 (103; 0-360)	36.9 (39.5; 0-100)

Table 2. Principal components and associated eigenvalues.

Principal Component	% total variance	% cumulative variance	Eigenvalue
PC1	0.270	0.270	3.5127
PC2	0.242	0.512	3.1412
PC3	0.175	0.687	2.2774
PC4	0.134	0.821	1.7390
PC5	0.073	0.894	0.9469
PC6	0.044	0.938	0.5725
PC7	0.027	0.965	0.3527
PC8	0.016	0.981	0.2102
PC9	0.008	0.989	0.1028
PC10	0.006	0.995	0.0826
PC11	0.003	0.998	0.0388
PC12	0.001	0.999	0.0135
PC13	0.001	1.000	0.0097

Table 3. Loading values for each principal component and the loading sum for each variable.

Variable	PC4	PC4*12.8	PC5	PC5*24.7	PC6	PC6*(-16.2)	Sum
Wind Speed, Mean	-0.4600	-5.8880	-0.1400	-3.4580	0.0030	-0.0486	-9.3946
Wind Speed, Min	-0.5230	-6.6944	-0.0610	-1.5067	0.5880	-9.5256	-17.7267
Wind Speed, Max	-0.3310	-4.2368	-0.0400	-0.9880	-0.7190	11.6478	6.4230
Temperature, Mean	0.3390	4.3392	0.0920	2.2724	0.0620	-1.0044	5.6072
Temperature, Min	0.3190	4.0832	0.0600	1.4820	0.1370	-2.2194	3.3458
Temperature, Max	0.3140	4.0192	0.1140	2.8158	-0.0170	0.2754	7.1104
Dew Point, Mean	-0.1250	-1.6000	-0.0200	-0.4940	-0.0590	0.9558	-1.1382
Dew Point, Min	-0.1430	-1.8304	0.0240	0.5928	-0.0220	0.3564	-0.8812
Dew Point, Max	-0.0540	-0.6912	-0.0690	-1.7043	-0.1090	1.7658	-0.6297
Sky Cover, Mean	0.0850	1.0880	-0.1160	-2.8652	0.0840	-1.3608	-3.1380
Sky Cover, Min	-0.0030	-0.0384	-0.0070	-0.1729	0.2610	-4.2282	-4.4395
Sky Cover, Max	0.0870	1.1136	-0.0730	-1.8031	-0.1540	2.4948	1.8053
Illumination	0.1900	2.4320	-0.9620	-23.7614	0.0130	-0.2106	-21.5400

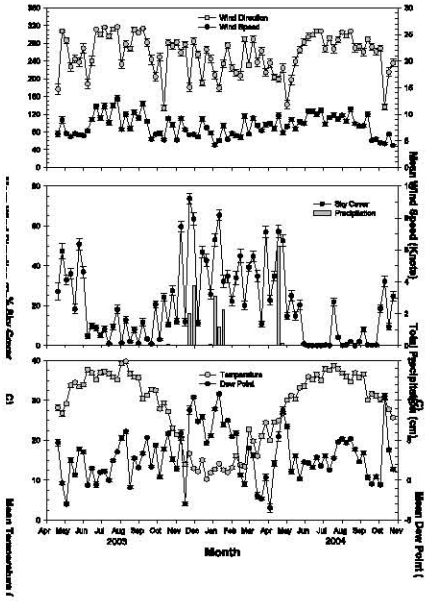

Figure 1. Seasonal variation in six meteorological parameters recorded at Tallil Air Base, Iraq from April 2003 through October 2004. Average weekly values are provided

(± SEM).

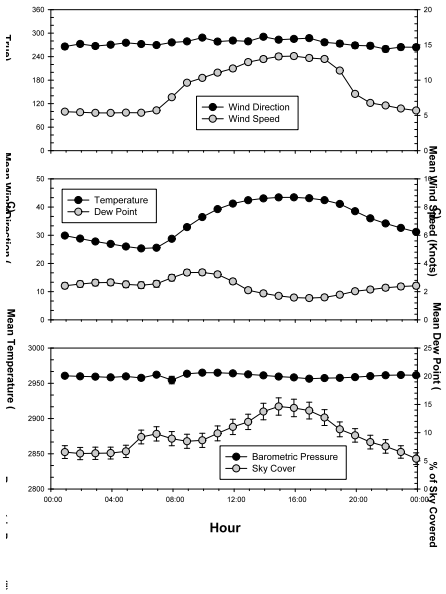

Figure 2. Daily variation in six meteorological parameters recorded at Tallil Air Base, Iraq during the hot season (May thru September) in 2003 and 2004. Points represent mean values (± SEM) recorded each hour over the entire hot season of both years.

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Chapter 3

Ecological niche model of *Phlebotomus alexandri* and *P. papatasi* (Diptera: Psychodidae) in the Middle East

Abstract

Ecological niche modeling (ENM) uses species presence records, such as museum collections and reports in the literature, and environmental layers to develop models of a species distribution. ENM techniques are increasingly being employed to understand the ecology of disease vectors. This study utilizes a maximum entropy (MaxEnt) approach to develop an ENM model of the distribution of two sand fly vectors of leishmaniasis, *Phlebotomus papatasi* (Scopoli) and *P. alexandri* (Sinton), across the Middle East. Collection records were obtained from literature reports from 1950 through 2007 and unpublished field collection records. Environmental layers considered in the model were elevation, precipitation, land cover, and bioclimatic variables reflecting different aspects of temperature and precipitation. The bioclimatic and altitude variables all contributed to model development; however, none influenced the model as strongly as land cover. While not perfect representations of the absolute ranges of *P. papatasi* and *P. alexandri*, these models indicate areas with a higher probability of presence of these species. This information could be used to help guide future research efforts into the ecology of these species and epidemiology of the pathogens that they transmit, disease prevention programs, and vector control efforts

Keywords: ecological niche model, *Phlebotomus*, sand fly, ecology, leishmaniasis

Phlebotomus (Phlebotomus) papatasi (Scopoli) and *P. (Paraphlebotomus) alexandri* (Sinton) are widely distributed across parts of Europe, Africa, and Asia. *Phlebotomus papatasi* is a vector of sand fly fever virus and *Leishmania major*, which causes cutaneous leishmaniasis (Ashford 2001a, b, Yaghoobi-Ershadi et al. 2005). *Phlebotomus alexandri* is a vector of *L. donovani* and is a suspected vector of *L. infantum*, both of which cause visceral leishmaniasis (Sukkar et al. 1985, Guan et al. 1986, Azizi et al. 2006). Though these species are important disease vectors, little is known about the ecology and distribution of each.

Phlebotomus papatasi ranges from Morocco and Spain, across the Mediterranean Basin to India and south to parts of the Sudan and Ethiopia (Lewis 1982). *Phlebotomus papatasi* is most abundant in areas with a mean minimum temperature of 16°C and mean maximum temperature of 44°C from May to October (Cross et al. 1996). It can be found at altitudes ranging from near sea level to over 1100 m (Belen et al. 2004).

Phlebotomus alexandri ranges from Spain and Morocco east to the mountains in northwestern China and as far south as southern Ethiopia (Depaquit 1997). This species has been recorded at altitudes ranging from sea level to 1500 m above sea level (Maroli et al. 2001, Kamal et al. 2003). In Djibouti, this species is found on the coastal plain, inland plateau, and highland valleys (Fryauff et al. 1995).

Characterizing the distribution and ecology of these vector species would be valuable to better understand the epidemiology of sand fly fever and leishmaniasis. Cross et al. (1996) developed a model of *P. papatasi* distribution in Southwest Asia based on weather and the normalized difference vegetation index (NDVI); however, the model was

not validated. Since the study was completed, powerful presence-only modeling techniques and software have been developed.

These newer modeling methods include a technique known as ecological niche modeling (ENM). ENM uses presence data in conjunction with environmental data to develop models of habitat range for a given organism (Peterson 2006). It is often used to examine the distribution of species that have not had intense, methodological sampling. This technique has been used in modeling distribution of diseases, such as dengue, and vectors, such as *Anopheles gambiae* (Costa et al. 2002, Peterson and Shaw 2003, Levine et al. 2004, Peterson et al. 2004, Peterson et al. 2005). These techniques have been used to examine the distribution and potential distribution of *Lutzomyia* spp. vectors of leishmaniasis in South America (Peterson and Shaw 2003, Peterson et al. 2004, da Costa et al. 2007).

In this study, we use ENM to develop distribution models for *P. papatasi* and *P. alexandri* in the Middle East. Using these models, we attempted to identify environmental factors that influence the distribution of these species.

Materials and Methods

Study Area. The study area included all or part of the following countries: Afghanistan, Armenia, Azerbaijan, Cyprus, Egypt, India, Iran, Iraq, Israel, Jordan, Kuwait, Kyrgyzstan, Lebanon, Oman, Pakistan, Qatar, Saudi Arabia, Syria, Tajikistan, Turkey, Turkmenistan, the United Arab Emirates, Uzbekistan, and Yemen (Figure 1). The coordinates delineating the corners of the study area were: northwest corner N 42.0819, E 25.4443; southwest corner N 11.3508, E 25.443; northeast corner N 42.0819, E 75.0586; southeast corner N 11.3508, E 75.0586.

Species Records. Presence data for the species were taken from records in the scientific literature dating from 1950 through 2007, and collections were performed by U.S. military entomologists in Iraq and Afghanistan between 2003 and 2006, and in Turkey in 2006. All coordinates were converted to the decimal degrees format. Records for which there was uncertainty about the location of the sampling or no specific location (i.e. a province given rather than a village for the collection) were excluded. For *P. papatasi*, 90 presence records were entered into the MaxEnt program. Of these, 68 were randomly selected for model development, with the remaining 22 used to test the model. For *P. alexandri*, 80 presence records were entered into the MaxEnt program. Sixty of these records were randomly selected for model development, with the remaining 20 used to test the model.

Environmental Layers. Climate and altitude layers were obtained from the WorldClim database, version 1.4 (www.worldclim.org). This database provides climate layers at a spatial resolution of 1 km^2 and is derived from weather station data from 1950-

2000 (Hijmans et al. 2005). For the purposes of this study, the WorldClim bioclimatic variables were used. These are listed in Table 1.

Land use data were obtained from the U.S. Geological Survey's (USGS) Earth Resources Observation and Science (EROS) Data Center (http://edc.usgs.gov/products/landcover/glcc.html). This is a global land cover classification that is broken into 96 land cover classes at 1-km resolution. Of the ninety-six classes, only 60 occur in the study area. The 68 training points for *P. papatasi* fall into just 10 of these classes (Table 2). The 60 training points for *P. alexandri* also are associated with 10 classes (Table 2), two of which were different from the classes associated with *P. papatasi* records.

Model Building and Evaluation. The niche modeling application MaxEnt version 3.2.1 was used in this analysis (Phillips et al. 2004). The MaxEnt program develops models of species distribution but are subject to the environmental variables that are entered into the model building process, using the principles of the maximum entropy distribution (Phillips et al. 2006, Phillips and Dudik 2008).

Seventy-five percent of the data points for each species were randomly selected as training points and were used in model building. The remaining 25% of the records were test points, used in model validation. Duplicate presence records were removed by the MaxEnt program prior to model development. The MaxEnt model output was set to logistic, which returns an estimated probability of presence for a given location between the values of 0 (no probability of species presence) and 1 (species is certain to be present). All other parameters were set to the default settings.

The model was evaluated using both threshold-dependent and threshold-independent methods. The area under the curve (AUC) of the receiver operating characteristic (ROC) analysis is a threshold-independent method of evaluating model quality. This technique computes the total AUC created by plotting sensitivity against the fractional predicted area for the species (Phillips et al. 2006, Pearson et al. 2007, Phillips and Dudik 2008). The threshold-dependent measure used here is the minimum training presence in which the probabilities are converted to binomial values with 0 being absent and 1 being present (Phillips et al. 2006, Pearson et al. 2007, Phillips and Dudik 2008). Using this method, all pixels with a probability of presence equal to or greater than that of the training point with the lowest probability of presence are classified as present, and all pixels with a lower probability of presence are classified as absent. A one-tailed binomial test is then performed with the null hypothesis being that the model does not predict the test points better than random (Phillips et al. 2006).

In order to determine which variables contribute most to the model development, the MaxEnt program was set to calculate jackknife tests of variable importance. The jackknife procedure produces three different types of models: (1) models created with one variable at a time excluded and all other variables included, (2) models created with only one variable included, and (3) a model created with all variables (Phillips et al. 2006, Pearson et al. 2007, Phillips and Dudik 2008). Variables that are most important to model development are those that decrease the training gain when removed from the model and show gain when the model is developed with only one variable.

Results

Phlebotomus papatasi

The MaxEnt model for *P. papatasi* is shown in Figure 1. The AUC for the training points was 0.944 and for test points was 0.884, with a standard deviation of 0.042. The minimum training presence for a training point was 0.197; therefore, this was set as the threshold for binomial conversion. The fractional predicted area (the area coded as 1 = present) is 0.346, and the omission rate for test points was 0.091. At this threshold, the test points were classified significantly better by the model than would be expected from random ($p<0.0001$).

Jackknife tests of variable importance show that land cover was the most influential variable in model development (Figure 2). The training gain when land cover was the only variable used in model development was high, indicating that it contributes strongly to the model. When land cover was removed from the model, training gain dropped. This indicates that the land cover variable contains unique information that is required for model creation. The land cover types and probabilities associated with the training points are given in Table 2. Points classified as urban, field/woody savanna, and woody savannah coverage are associated with high probabilities of presence. However, the sample size is small for both field/woody savanna and woody savanna. Points classified as bare desert have low probabilities of presence; however the sample size is small. All other classes have either very wide ranges or a sample size of one.

The remaining variables contributed much less to model development (Figure 2). Isothermality (Bio3), maximum temperature in the warmest month (Bio5), minimum

temperature in the coldest month (Bio6), mean temperature in the coldest quarter (Bio11), precipitation seasonality (Bio15), and precipitation in the coldest quarter (Bio19) all had modest gains when run with only the variable in question. However, the model gain was minimally decreased by the exclusion of the variables from the analysis. This is a reflection of the correlated nature of the bioclimatic variables and an indication that none of these variables in isolation are overwhelmingly contributing to this model.

Phlebotomus alexandri

The MaxEnt model for *P. alexandri* is shown in Figure 3. The training AUC was 0.942 and the test AUC was 0.844, with a standard deviation of 0.044. The minimum training presence among training points was 0.164. At this threshold, the fractional predicted area was 0.368 and the omission rate for test points was 0.200. The model correctly classifies the test points as significant more than a random model ($p < 0.0001$).

As in the model for *P. papatasi*, land cover was the most influential variable in modeling *P. alexandri* (Figure 4). Jackknife tests show high training gain when land cover is considered alone and a large drop in training gain when land cover is omitted from the model. The land cover types and probabilities associated with the training points are given in Table 2. As with *P. papatasi*, points classified as urban, field/woody savanna, and woody savanna coverages have high probabilities of presence. However, the sample sizes for each of these habitats are small. All other classes have either very wide ranges or small sample size.

The 19 bioclimatic variables and elevation all have very modest training gains when considered in isolation (Figure 4), indicating that none of them strongly contribute to model development on their own. Elevation, min temperature in the coldest month

(Bio6), mean temperature in the wettest quarter (Bio8), and precipitation in the driest month all show modest decreases in training gain when removed from the model. This indicates that they may contain unique information required for the model.

Discussion

In this study, land cover contributed strongly to the development of the models for both species. The urban land cover class was associated with a high probability of presence for both species. This may be partly due to sampling bias, as collections of phlebotomine sand flies tend to be associated with research related to human leishmaniasis. However, a survey of sand flies in Sanliurfa Province, Turkey, *P. alexandri* was present in the more urban areas of the province, but not in the rural areas (Toprak and Ozer 2007). *Phlebotomus alexandri* is anthropophilic. It is possible that this feeding relationship would drive the species to be more probable in urban environments simply because humans are more readily available. While there may be a relationship between these species and urban environments, the magnitude of that relationship seen here may be biased by the nature of sand fly and leishmaniasis research.

Other non-urban land cover types are also important for these species. In the present study, the logistic probability of presence of both species for the points that fall in the woody savannah and field and woody savanna classes are high (mean 0.9278 and 0.7955, respectively), though the sample sizes are small. The range of probability of presence for both species in relation to semi-desert shrub is extremely wide for both species (Table 2). This makes it difficult to draw any conclusions about this particular coverage class in this project. However, research in Morocco has shown that both species are associated with desert, scrub vegetation (Rispail et al. 2002).

For *P. papatasi*, bare desert appears to be related to a low probability of presence in this study, though only five training points were located in this cover class. In Israel,

P. papatasi is more abundant in areas with more humid soils, capable of supporting desert vegetation, than in areas with low soil moisture and less vegetation (Wasserberg et al. 2002, Wasserberg et al. 2003). Sand flies require a sugar meal, taken from plant material (El Said et al. 1986, Schlein and Jacobson 1999); therefore, they would be expected to be less abundant in areas with little or no vegetation, such as a barren desert.

Cross et al. (1996) used normalized difference vegetation index (NDVI) and weather data to develop a predictive model of *P. papatasi* distribution in southwest Asia; however the models were not validated. We considered including 1-kilometer resolution NDVI data in this model; however, the addition of NDVI to the model development did not affect the outcome (unpublished data). When land cover was excluded and NDVI and the bioclimatic variables were used for model building, the resulting product did not perform as well as the models presented here (unpublished data). While NDVI may help model development in some cases, 1-km resolution NDVI data taken from Advanced Very High Resolution Radiometer (AVHRR) sensors were not as useful as other environmental and climatic variables in this case.

Temperature and precipitation are important for model development but were not the leading factor in the development of the models presented here. In Morocco, sand flies are most active in the hot, dry season, with *P. papatasi* most abundant when ambient temperature is in the 32-36^{o}C range (Boussaa et al. 2005). In Pondicherry, India, *P. papatasi* reaches its peak abundance at the end of monsoon season (Srinivasan et al. 1993). Sand flies in Oman, particularly *P. alexandri*, are more abundant during periods of low humidity and high temperature (Roberts 1994). In the present study, the bioclimatic variables all contributed toward model development. However, none of these

variables were particularly valuable in isolation for either model creation or validation. This is a reflection of the correlated nature of the temperature and precipitation variables.

These models are estimates of the distribution of *P. papatasi* and *P. alexandri*, based on the environmental layers chosen in the study and the location of collection records. As such, they are not a definitive guide as to whether or not a species will be present in a given area. However, they can be used to estimate to the probability that *P. papatasi* and/or *P. alexandri* are present in an area. Since animal reservoir, *Leishmania* parasite, and/or leishmaniasis disease data are not included in this work, these models do not reflect the distribution of disease. Future models incorporating records of *Leishmania* parasites, disease, and reservoir populations could provide a better understanding of the distribution and risk of cutaneous and visceral leishmaniasis transmission in this area. In addition, expansion of the scope of the modeling effort to include the entire range of these species may further refine the models.

Table 1. WorldClim variables and the mean and ranges of each variable for the presence points for *Phlebotomus papatasi* and *P. alexandri* used in this study.

Variable	Description (http://www.worldclim.org/bioclim.htm)	*P. papatasi* Mean (range)	*P. alexandri* Mean (range)
Bio1	Annual mean temperature, ^{o}C	19.73 (9.50 – 27.50)	20.79 (10.80 – 30.70)
Bio2	Mean diurnal range (Mean of monthly (max temp – min temp)), ^{o}C	13.79 (8.20 – 17.20)	13.57 (7.00 – 17.20)
Bio3	Isothermality ((Bio2/Bio7)*100), ^{o}C	3.90 (3.00 – 6.20)	4.09 (3.00 – 6.20)
Bio4	Temperature Seasonality (standard deviation *100), ^{o}C	771.85 (263.80 – 986.00)	712.96 (263.80 – 959.20)
Bio5	Max temperature of the warmest month, ^{o}C	38.01 (27.20 – 44.20)	38.25 (28.70 – 46.00)
Bio6	Min temperature of the coldest month, ^{o}C	2.75 (-8.30 – 15.10)	4.81 (-8.20 – 22.70)
Bio7	Temperature annual range (Bio5 – Bio6), ^{o}C	35.26 (19.70 – 42.80)	33.44 (17.60 – 42.80)
Bio8	Mean temperature of the wettest quarter, ^{o}C	13.19 (0.70 – 33.60)	14.27 (1.60 – 36.90)
Bio9	Mean temperature of the driest quarter, ^{o}C	27.72 (14.40 – 35.90)	28.18 (15.60 – 35.80)
Bio10	Mean temperature of the warmest quarter, ^{o}C	29.24 (18.50 – 35.90)	29.65 (21.20 – 36.90)

Bio11	Mean temperature of the coldest quarter, ^{o}C	9.52 (-2.00 – 21.40)	11.42 (-2.00 – 26.10)
Bio12	Annual precipitation, mm	323.64 (21.00 – 1255.00)	214.06 (21.00 – 795.00)
Bio13	Precipitation of the wettest month, mm	68.71 (4.00 – 250.00)	44.69 (4.00 – 166.00)
Bio14	Precipitation of the driest month, mm	1.61 (0.00 – 29.00)	0.68 (0.00 – 7.00)
Bio15	Precipitation seasonality (coefficient of variation), mm	88.59 (38.00 – 161.00)	83.56 (37.00 – 151.00)
Bio16	Precipitation of the wettest quarter, mm	177.39 (11.00 – 619.00)	114.66 (11.00 – 435.00)
Bio17	Precipitation of the driest quarter, mm	8.53 (0.00 – 120.00)	5.33 (0.00 – 52.00)
Bio18	Precipitation of the warmest quarter, mm	28.21 (0.00 – 569.00)	14.20 (0.00 – 126.00)
Bio19	Precipitation of the coldest quarter, mm	142.48 (8.00 – 619.00)	93.78 (0.00 – 126.00)
Alt	Altitude (elevation above sea level), m	606.42 (-372.00 – 2440.00)	676.78 (-81.00 – 2440.00)

Table 2. Land cover classes associated with training points for *Phlebotomus papatasi* and *P. alexandri*.

	Probability of Presence					
	Phlebotomus papatasi			*Phlebotomus alexandri*		
Land Cover Class	n*	Mean**	Range***	n	Mean	Range
Bare desert	5	0.2614	0.1852 - 0.3473	8	0.5708	0.0894 - 0.9724
Crops and town	6	0.6965	0.1798 - 0.9587	--	--	--
Crops, grass, and shrub	--	--	--	1	0.6880	--
Dry woody scrub	--	--	--	1	0.9791	--
Field/woody savanna	5	0.7955	0.6827 - 0.9383	5	0.8509	0.7250 - 0.9066
Grass crops	6	0.6958	0.2110 - 0.9427	3	0.5313	0.4324 - 0.6470
Hot, irrigated cropland	1	0.6714	--	--	--	--
Irrigated grassland	1	0.3477	--	1	0.4683	--
Low sparse grassland	4	0.3754	0.1729 - 0.6176	1	0.2610	--
Semi-desert shrubs	18	0.5220	0.2258 - 0.7222	27	0.6913	0.2099 - 0.9042
Urban	20	0.9576	0.7012 - 0.9915	10	0.9667	0.8889 - 0.9933
Woody savanna	2	0.9278	0.9174 - 0.9382	2	0.9191	0.8883 - 0.9500

* n = the number of training points that occurred in a given land cover class

** Mean is the mean probability of the species being present among points that occur in a given land cover class

*** Range is the range of probability values associated with the points that occur in that land cover class

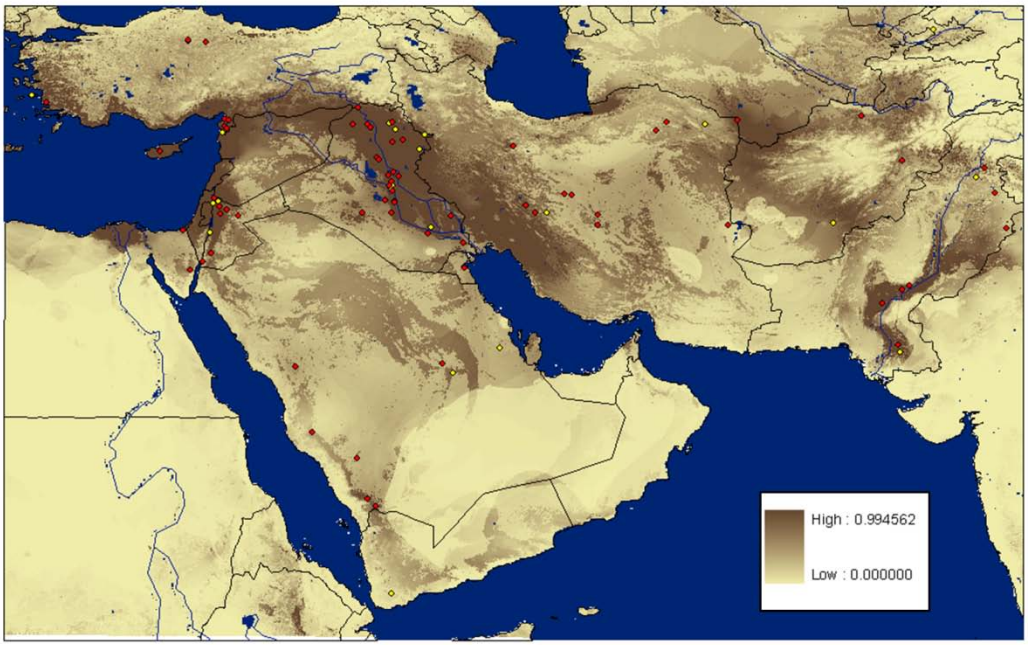

Fig. 1. Predicted distribution of *Phlebotomus pap*atasi in the Middle East. Lighter areas indicate low probability of occurrence, darker areas indicate high probability of occurrence. Red points indicate training records and yellow points indicate test records.

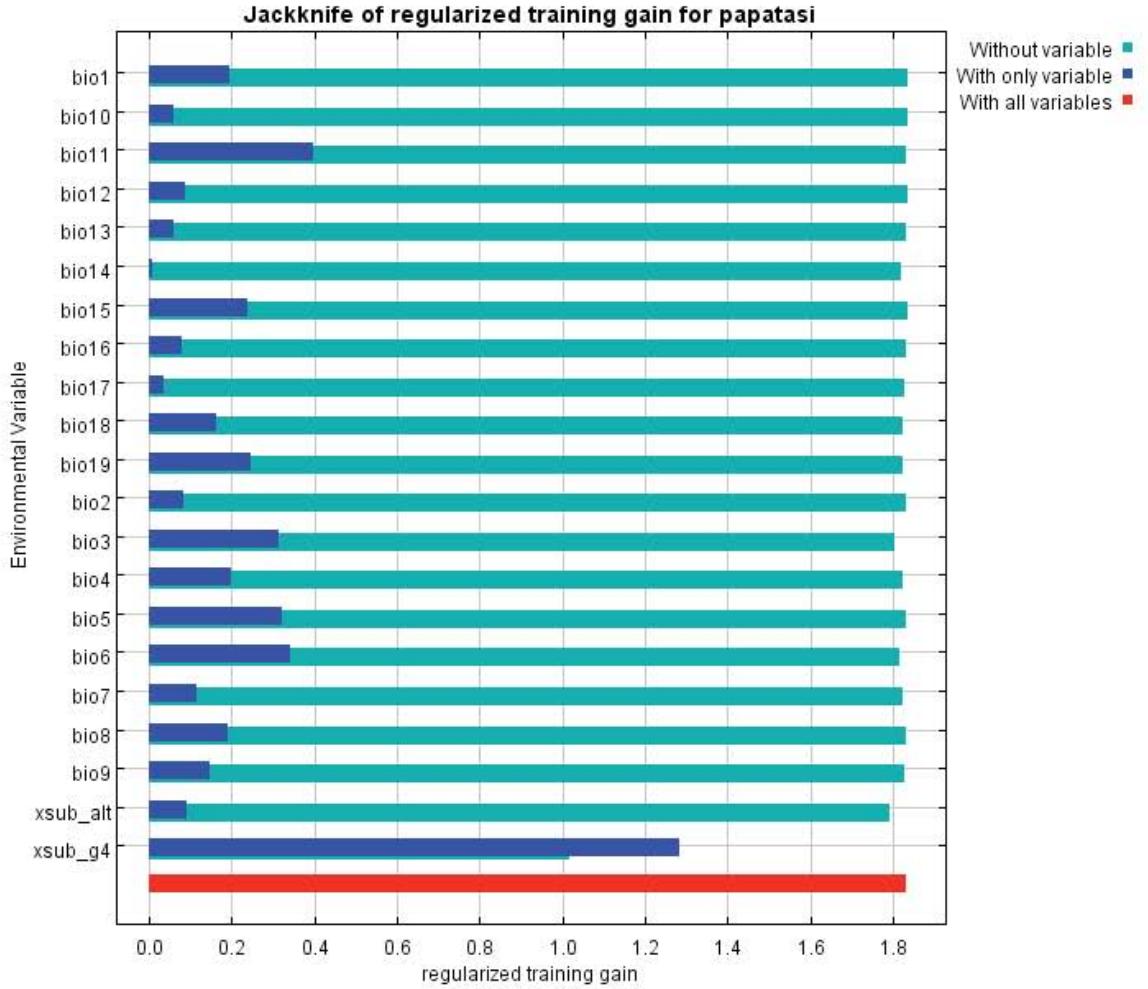

Figure 2. Jackknife test of training gain for *P. papatasi*. Environmental variables: Bio1through Bio 19 represent the bioclimatic variables (Table 1); xsub_alt is the altitude layer; xsub_g4 is the land cover layer.

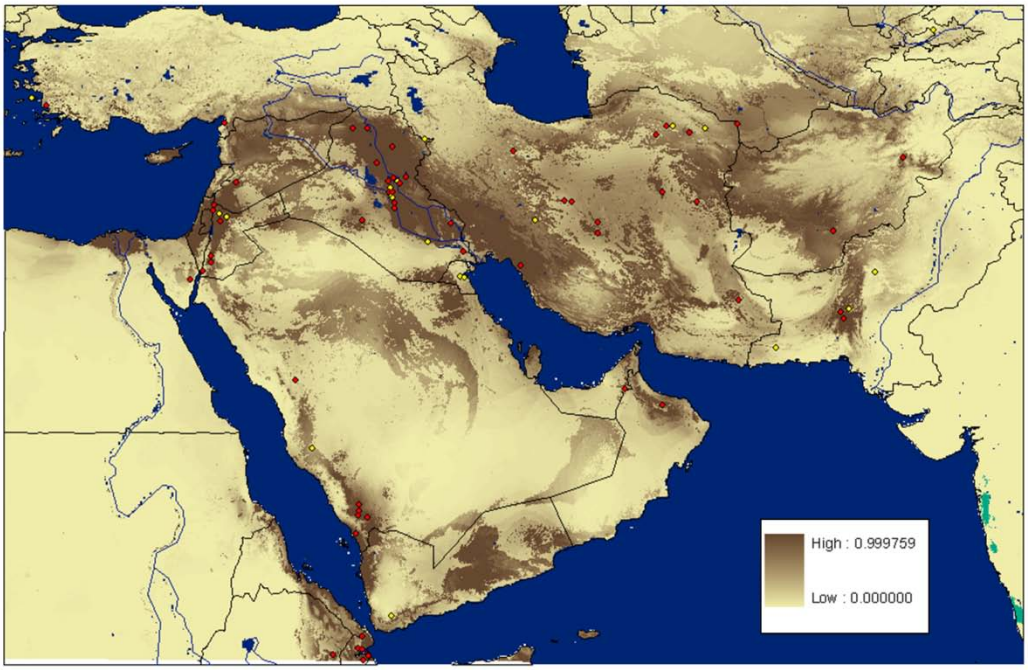

Fig. 3. Predicted distribution of *Phlebotomus alexandri* in the Middle East. Lighter areas indicate low probability of occurrence, darker areas indicate high probability of occurrence. Red points indicate training records and yellow points indicate test records.

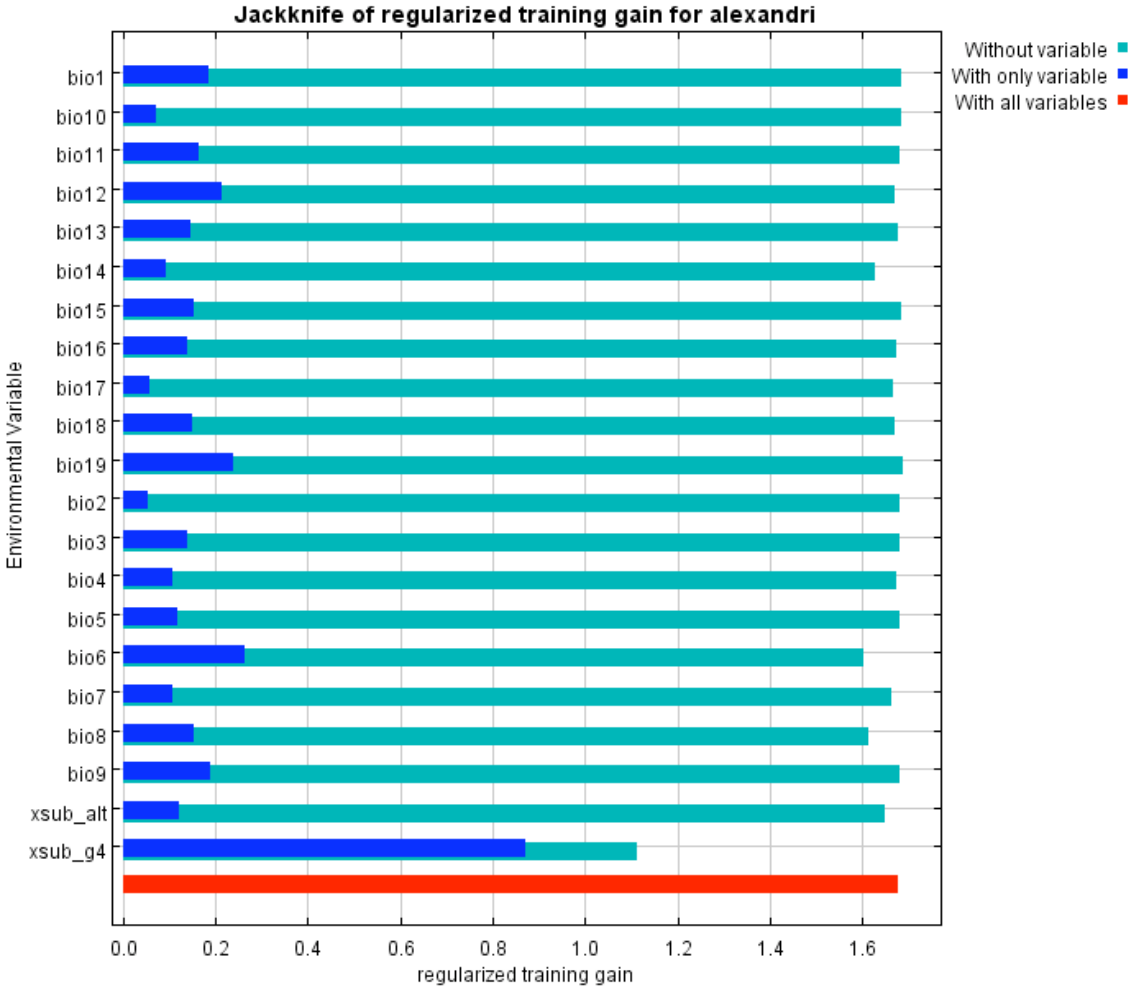

Figure 4. Jackknife test of training gain for *P. alexandri*. Environmental variables: Bio1through Bio 19 represent the bioclimatic variables (Table 1); xsub_alt is the altitude layer; xsub_g4 is the land cover layer.

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Chapter 4

Examination of Normalized Difference Vegetation Index and *Phlebotomus papatasi* collection in Iraq

Abstract

The goal of this study was to determine whether Normalized Difference Vegetation Index (NDVI) is related to the population levels of the sand fly, *Phlebotomus papatasi* as measured by light trap collections. Moderate Resolution Imaging Spectroradiometer (MODIS) NDVI values for October 2004 – September 2005 were compared to sand fly collection data from U.S. military entomologists in Iraq from April to May 2005. The original NDVI images were processed with a mean value filter and maximum value filter, both with a 1 km radius. The twelve original images were also averaged and the averaged image was processed with the mean and maximum filters. Spearman rank correlations were calculated for all layers derived from the original twelve images and each month of sand fly collection date. There were no significant correlations between the three average NDVI layers and any *P. papatasi* in any collection month. There were also no significant correlations found for the 250-m resolution layers. For the maximum filter, there were significant ($p<0.05$) correlations between the May and September collection months and the winter NDVI values. For the mean filter, there were significant correlations between the September collections and the winter NDVI values. Further research needs to be conducted to fully evaluate the appropriate scale of NDVI data required for use with sand flies.

Normalized difference vegetation index (NDVI) is one of several different vegetation indices that characterize the vegetation in an area from remotely sensed satellite imagery. It has been used to predict habitat suitability for disease vectors and to help predict outbreaks of vector borne diseases. NDVI has been used to classify habitat suitability for *Culicoides spp.*, vectors of bluetongue virus (Calvete et al. 2008); various tick species (Estrada-Pena and Thuiller 2008); and *Anopheles spp.* mosquitoes (Ceccato et al. 2007).

In addition to characterizing habitat suitability, NDVI is used to help develop models that can aid in predicting disease outbreaks. Research into indicators of Rift Valley Fever outbreaks has shown that NDVI is an early warning indicator of the potential for epidemics (Linthicum et al. 1999, Anyamba et al. 2002). This model was successfully used to predict an epidemic of Rift Valley Fever in 2006 – 2007 (Indeje et al. 2006). As NDVI has been shown to be a valuable tool in predicting habitat suitability for disease vectors and disease outbreaks, there is interest in applying this tool for other disease vector systems.

Sand flies (Diptera: Psychodidae) in the genus *Phlebotomus* are vectors of leishmaniasis and sand fly fever in the Old World. Cross et al (1996) used NDVI and temperature were used to develop a model of the distribution of *Phlebotomus papatasi*, a vector of cutaneous leishmaniasis and sand fly fever in Southwest Asia, though this model was never validated. Previous work by the authors of the present study included 1-km resolution NDVI data in an ecological niche model (ENM) of *Phlebotomus alexandri,* vector of visceral leishmaniasis, and *P. papatasi* in the Middle East; however, NDVI did not contribute to the resulting model (Colacicco et al. 2009). Based on the

work by Cross et al. (1996) and the applications of NDVI to other vector species, this was a surprising finding.

The authors hypothesize that the lack of contribution by NDVI to their previous modeling efforts was likely due to the choice of 1-km resolution NDVI imagery and that a higher resolution may be a better choice in the case of sand flies. In this study, we examine the relationship between *P. papatasi* and 250-m resolution NDVI imagery.

Materials and Methods

Sand fly collections

U.S. military entomologists have been collecting sand flies in Iraq as part of the Leishmaniasis Control Program since 2003 (Coleman et al. 2006). In the present study, we examined collection records of *P. papatasi* collected in Iraq from April – September 2005. All sand fly collections were performed using standard, unbaited CDC light traps. Sand flies were shipped from Iraq to the Walter Reed Army Institute of Research (WRAIR) in Silver Spring, Maryland for identification. Collection records were separated by month and the number of trap nights was calculated for each unique sampling location in a given month.

Satellite imagery

We obtained 250-m resolution Moderate Resolution Imaging Spectroradiometer (MODIS) NDVI imagery for October 2004 – September 2005 from the Level 1 and Atmospheric Archive and Distribution System (LAADS) Web, which is maintained by the National Aeronautic and Space Administration's (NASA) Goddard Space Flight Center in Greenbelt, Maryland (http://ladsweb.nascom.nasa.gov/). The name of the data set is "Vegetation Indices 16-day L3 Global 250 m," also referred to as MOD13Q1. The product provides a 16 day composite image at a 250 m spatial resolution. For all months with more than one set of images, the first 16-day composite for each month was used in this study.

Data extraction and statistical analysis

The NDVI layers and the monthly sand fly collection records were imported into ArcGIS, version 9.2. In order to account for the sand fly sampling occurring in and around developed areas where there is less potential for vegetation, the NDVI layers were filtered to include surrounding pixel values. The filters were circle shaped around each point, with a radius of 4 cells or 1 km. For each of the original NDVI images, a maximum value and mean value filter was applied. The maximum value filter extracts the highest NDVI value within the 1 km radius circle around the sampling location. The mean value filter calculates the mean NDVI of the area within the filter from the original image. This resulted in a total of 36 NDVI layers (250-m resolution, mean filter, and maximum filter for each month). Figure 1 shows the NDVI image for December 2004 at the original 250 m resolution and with the mean and maximum filters applied.

In order to examine whether there was a relationship between the entire 12-month data set and *P. papatasi,* the twelve original 250 m resolution NDVI layers were averaged. ArcGIS was used to average the layers, which is done by averaging the value for each pixel across the 12 original images. The mean and maximum filters were then run on this averaged layer in the manner previously described.

Collection records for the 2005 field season were overlaid on each of the 36 NDVI layers. The NDVI value corresponding to the location of each light trap was extracted for each layer. These values were then exported to SAS software, version 9.1, for statistical analysis.

Since the collection data is not normally distributed, Spearman's rank correlation was used to determine any significant correlations between the sand fly collection data

and NDVI (Zar 1999). For the monthly NDVI products (250 m resolution, mean filter, and max filter), correlations were determined between the collection values for each month and the NDVI for each preceding month. Spearman's rank correlation was also used with the averaged NDVI at the 250m resolution, mean filtered data, and maximum filtered data. A significance level of 0.05 was used to evaluate the strength of the correlations.

Results

The number of collection locations varied over the course of the April 2005 – September 2005 collection season. During the 2005 collection season, light traps were placed in 62 unique locations. Of these, 26 sites were only used in one month. Five sites were sampled for 5 months of the season. In all months except for September, there were some light traps at which *P. papatasi* was not collected during the month (Table 1). The mean NDVI values for all sampling sites for each month are given in Table 2. The lowest NDVI value at a sampling site was 0.037 in December 2004. The highest NDVI value recorded from a sampling site was 0.4629, occurring in March 2005.

For the 250-m resolution NDVI layers, the only significant correlation between *P. papatasi* collection and NDVI was between the July 2005 NDVI value and the collection for that same month, with a correlation coefficient of 0.39318 ($p < 0.05$). For the NDVI max filter, there were several significant correlations (Table 3). There is a direct relationship between the June NDVI data and the June collection data. The significant inverse correlations between the May and September sand fly collections and the winter NDVI values are particularly interesting. A similar trend is seen with the NDVI mean filter (Table 4).

When the twelve original NDVI layers were averaged, the lowest NDVI value for a sampling site was 0.0779 and the maximum was 0.2997 (mean=0.1268). There were no significant correlations between the 250m resolution average NDVI layer and *P. papatasi* collections in any of the sampling months. There were also no significant correlations between collections in a given month and the 12 month average NDVI with the

maximum filter. Finally, there were no significant correlations when the mean filter was applied.

Discussion

In addition to the requirement of female sand flies for a blood meal, all sand flies require a sugar source for survival (Schlein and Jacobson 1999). In order to obtain this sugar meal, they require some association with vegetation. In addition to being attracted to plants upon which they feed, *P. papatasi* is also attracted to plants from which they will not feed, possibly for shelter or breeding sites (Schlein and Yuval 1987). One study in the Sudan of the relationship between several sand fly species and vegetation found that *P. papatasi* was most abundant in and around villages, where the vegetation was not very dense (Elnaiem et al. 1999).

The correlations between the May and September *P. papatasi* collections and the winter NDVI values are particularly interesting. Work in the Jordan Valley has shown that increased *P. papatasi* populations correspond with irrigated agricultural land (Yuval 1991, Janini et al. 1995). In that area, *P. papatasi* are strongly associated with the fat sand rat, *Psamomys obesus*, which has its burrows around plants in the family Chenopodiaceae. The increased *P. papatasi* populations are likely due to an increase in the number of Chenopod plants supporting larger populations of the fat sand rat (Janini et al. 1995). In a comparison of different sites in Nizzana, Israel, *P. papatasi* was positively correlated with soil moisture (Wasserberg et al. 2002). The amount of moisture available to a plant in the soil could affect the NDVI level.

The lifecycle of *P. papatasi* is temperature dependent and may take anywhere from one to several months (Srinivasan and Panicker 1993, Kasap and Alten 2006). In many areas, populations decline steeply during the winter months. When the

environmental conditions are unfavorable for the development, *P. papatasi* can enter diapause in either the egg or fourth larval instar, resuming development when the conditions are once again favorable. *Phlebotomus papatasi* employs this tactic to overwinter, with the proportion of fourth instar larvae entering diapause reaching a peak in the fall (Rutledge and Gupta 2002).

The inverse relationship between sand fly *P. papatasi* population levels in May and the NDVI in the winter months (using the maximum filter) potentially reflects some indicator of overwintering site quality. The NDVI values may be related to factors which either inhibit or promote vegetation in an area, such as soil moisture, soil chemistry, or soil composition. If this is the case, the inverse relationship shown here may actually reflect a relationship with some other aspect of the environment.

The inverse relationship between the September collections and the winter NDVI values for both the maximum and mean filtered images are likely also a reflection of the relationship with overwintering conditions. The number of sand flies present in the late summer is dependent on the health and breeding success of the population earlier in the season. If the overwintering conditions are not favorable to the survival of diapausing *P. papatasi*, then the early season numbers would be adversely affected, which would then decrease the potential population later in the season.

Cross et al. (1996) developed a model of habitat suitability for *P. papatasi* in Southwest Asia using NDVI and temperature data. According to this study, the range of NDVI values suitable for *P. papatasi* was from 0.00 to 0.06. The minimum NDVI value associated with the presence of *P. papatasi* in the present study is 0.037 and the maximum was 0.4629. While this may appear to be a big discrepancy between the two

studies, the NDVI values cannot be directly compared because the satellites used to obtain the imagers have different sensors, which can affect the interpretation of the NDVI value. The model developed in that study used Advanced Very High Resolution Radiometer (AVHRR) NDVI, with 1 pixel approximately equal to 8 km^2; whereas, the present study uses 250m resolution NDVI. It would be instructive to examine this data set against the same AVHRR imagery used in the previous model to evaluate whether the differences in NDVI levels between the two studies are real.

A key consideration in research involving remote sensing technology is the scale or spatial resolution of the data. In a study aiming to determine if there NDVI can be used to explain ranging patterns of antelope, NDVI at a larger scale was required to detect the relationship (Bro-Jorgensen et al. 2008). In the case of Phlebotomine sand flies, it is likely that any relationship between NDVI and sand flies would require a fine resolution in the satellite imagery. In the study by Cross et al (1996) the authors blocked out a 3x3 pixel area around the individual point (one pixel = 7.6 km^2). NDVI was then determined from that area. In the present study, we used 250m resolution imagery and applied a 1km diameter circle filter around the points for the maximum and mean filters.

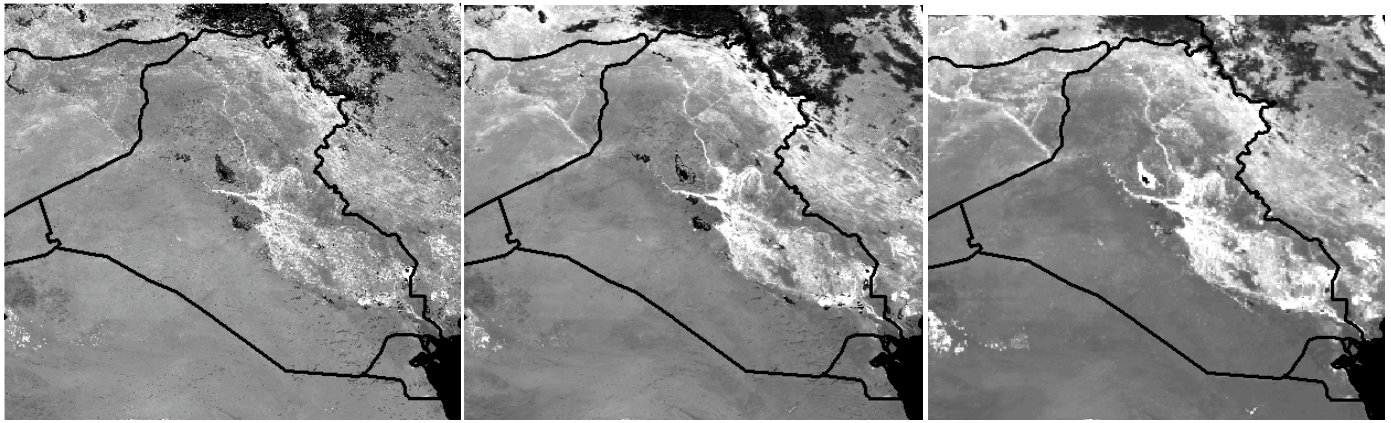
Sand flies are relatively weak fliers. They are most abundant when wind speeds are less than 0.3 m/s and the maximum wind speed against which they can fly is estimated at 3.5 m/s (Roberts and Kumar 1994, Sawalha et al. 2003). *Phlebotomus araisi* in France have been found to disperse approximately 1 km from their point of origin, with a flight speed of approximately 0.70 m/s under optimal conditions (Killick-Kendrick et al. 1985, Killick-Kendrick et al. 1986). For the same species, the distance of attraction to the type of CDC light trap used in the present study was found to be less than 2m

(Killick-Kendrick et al. 1985). Taking the work with *P. ariasi* as a model, it can be presumed that *P. papatasi* disperses apprixmately 1km and is attracted to light traps when they are within approximately 2m of the light. Given the weak nature of sand fly flight, the dispersal distance of the flies, and the attractive distance of a CDC light trap, the spatial scale for NDVI used to determine any correlation between sand flies captured using a light trap should be relatively small. This was the rational for the choice of the 250 m resolution imagery and the 1km filter used in the present study. While there were some correlations, it is possible that an even finer scale is required to identify whether a relationship is present.

There are a few issues particular to this project which may affect the ability of this study to detect relationships. First, the sand fly collections were made by U.S. military entomologists on camps occupied by U.S. forces. This was not a comprehensive survey across Iraq. Therefore, it is possible the findings are somewhat artificial. However, since *P. papatasi* is a public health concern in those areas where it interacts with human populations, this may be an acceptable bias. Secondly, this examination covers only one year of collection and NDVI data. The 2005 collection data was selected for consideration because the sampling effort was more uniform across Iraq than in previous years. However, inclusion of more than a year's worth of collection and NDVI would have helped rule out the possibility of either an anomaly in the sand fly collections or the NDVI values for the study period.

Future efforts to determine the applicability of NDVI in helping predict sand fly populations are required to shed more light on this issue. Multi-year analyses in areas where a systematic sampling effort can be conducted is necessary to better understand

any relationship that may be present. In addition, a systematic study looking at the relationship between *P. papatasi* and NDVI at different resolutions would be instructive in helping to determine the utility of NDVI as a tool to predict or explain sand fly populations.



A. B. C.

Figure 1. Normalized Difference Vegetation Index (NDVI) for Iraq in December 2004. A) The original 250 m NDVI; B) With a 1 km mean value filter on a 250 m resolution MODIS NDVI image; C) With a 1 km maximum value filter on a 250 m resolution MODIS NDVI image.

Table 1. Number of sights with traps each month of the 2005 collection season and the number of sites for each month with *Phlebotomus papatasi*.

Month	Number of Sites	Sites with *P. papatasi*
April	18	11
May	25	21
June	30	23
July	28	22
August	19	17
September	23	23

Table 2. 250-m resolution Normalized Difference Vegetation Index (NDVI) values by month across all light trap locations for the 2005 collection season.

		NDVI (250m resolution)		
Year	Month	Mean	Min	Max
2004	October	0.1277	0.0795	0.3317
	November	0.1515	0.0586	0.3734
	December	0.1429	0.0370	0.3869
2005	January	0.1415	0.0629	0.3465
	February	0.1494	0.0712	0.3584
	March	0.1665	0.0800	0.4629
	April	0.1543	0.0794	0.3178
	May	0.1292	0.0700	0.2777
	June	0.1155	0.0688	0.2741
	July	0.1174	0.0675	0.2725
	August	0.1242	0.0690	0.3584
	September	0.1263	0.0705	0.358

Table 3. Spearman's rank correlation of NDVI value (maximum filter) by month with the *P. papatasi* collection by month.

		NDVI – Max Filter											
		Oct 04	Nov 04	Dec 04	Jan 05	Feb 05	Mar 05	Apr 05	May 05	Jun 05	Jul 05	Aug 05	Sep 05
P. papatasi	Apr	-0.27326	-0.04489	0.03499	0.12826	0.32086	0.27173	-0.02534	----	----	----	----	----
	May	**-0.45324***	**-0.49653**	**-0.431213**	-0.35691	-0.16614	-0.15223	-0.37379	-0.39093	----	----	----	----
	Jun	-0.19355	-0.23274	-0.13617	-0.18419	0.17460	0.05950	-0.08678	-0.28379	**0.47876**	----	----	----
	Jul	0.19383	0.20934	0.22580	0.20212	0.02981	0.13826	0.26042	0.20881	0.16206	0.29440	----	----
	Aug	0.01290	-0.00089	-0.02801	-0.00178	-0.09956	-0.19716	-0.09467	0.13017	-0.18649	0.01022	-0.00888	----
	Sep	-0.40815	**-0.43462**	**-0.50714**	**-0.57566**	-0.35090	-0.17549	-0.26418	-0.25750	-0.05967	-0.36506	**-0.43365**	-0.23066

*Significant correlations ($p<0.05$) are in bold print.

Table 4. Spearman's rank correlation of NDVI value (mean filter) by month with the *P. papatasi* collection by month.

		NDVI – Mean Filter											
		Oct 04	Nov 04	Dec 04	Jan 05	Feb 05	Mar 05	Apr 05	May 05	Jun 05	Jul 05	Aug 05	Sep 05
P. papatasi	Apr	0.04087	0.15596	0.12692	-0.00430	0.30117	0.16242	0.17102	----	----	----	----	----
	May	**-0.40100***	-0.32581	-0.21969	-0.31524	0.11591	-0.06070	-0.19424	-0.32425	----	----	----	----
	Jun	-0.14967	-0.03595	0.11712	-0.08614	0.32060	0.19331	0.03278	-0.15827	-0.22677	----	----	----
	Jul	0.17073	0.11085	0.16432	0.09720	-0.12004	0.05375	0.18215	0.26933	0.30776	0.32754	----	----
	Aug	-0.13855	-0.10125	-0.12790	-0.19540	-0.11990	-0.15809	-0.9770	-0.08171	0.00178	-0.10658	-0.00888	----
	Sep	**-0.59284**	**-0.57388**	**-0.50801**	**-0.43315**	-0.37227	-0.27446	-0.31239	**-0.44962**	-0.41269	-0.41269	**-0.43365**	**-0.46060**

*Significant correlations ($p<0.05$) are in bold print.

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Chapter 5

Molecular analysis of the cytochrome b mtDNA and ITS2 rDNA sequences for *Phlebotomus alexandri* from sites in Iraq and Turkey

Abstract

The present study sought to examine the genetic similarity of populations of *Phlebotomus alexandri* at different points across its range. The results reported here represent part of a larger collaborative effort, which is not yet complete. In the present work, samples from five locations in Iraq and one location in Turkey were examined in this study in an effort to determine if the populations of *P. alexandri* are different. The samples from Iraq were collected from 2003 – 2007 by U.S. military entomologists in Iraq. The samples from Akbuk, Turkey were collected by a group of entomologists from the Walter Reed Institute of Research (WRAIR) in 2006. Cytochrome b (cyt b) and the second internal transcribed spacer (ITS2) were used in this study. The cytb DNA was successfully sequenced for 21 specimens. The ITS2 sequence was more difficult to sequence, with only 10 specimens being sufficiently sequenced. Phyllip version 3.68 was used to perform phylogenetic analysis. Bootstrap analysis, neighbor-joining distance analysis, maximum parsimony, and maximum likelihood techniques were employed to develop phylogenies. The basic phylogenies produced by all methods were similar. Based on the results, it appears that the Turkey and Iraq populations are different; however, there is no difference between populations in Iraq. Further work is required to determine whether or not populations of this species are significantly different across its range.

Phlebotomus (Paraphlebotomus) alexandri has a wide range in the Old World, extending from Spain and Morocco east to China. This species is a proven vector of *Leishmania donovani* (Guan et al. 1986). Additionally, it has been found to be infected with *Leishmania infantum* in Iraq and is considered a suspected vector, though it has not yet been demonstrated to transmit the pathogen (Azizi et al. 2006). While little research has been focused on *P. alexandri*, it has been speculated that the large range and morphological differences across the range may be indicators that this is not actually one species (Depaquit 1997, Kakarsulemankhel 2004).

Phlebotomus (Paraphlebotomus) sergenti, a closely related species, is the primary vector of *L. tropica* in the Middle East (Svobodova et al. 2007). Like *P. alexandri*, it has a wide distribution, ranging from Spain and Morocco to Central Asia (Depaquit 1997). Depaquit et al (2002) examined heterogeneity between populations of *P. sergenti* using the internal transcribed spacer 2 (ITS2). Some variation was detected, dividing the range of the species into two different populations that coincide with differences in the epidemiology of *L. tropica* (Depaquit et al. 2002). Moin-Vaziri et al (2007) found some variation in the cytochrome b (cyt b) gene of the mitochondrial DNA among populations of *P. sergenti* in Iran. Baron et al (2008) incorporated both the ITS2 and cyt b markers to identify differences between populations of *P. sergenti* from Spain and Morocco. It is possible that the genetic variation seen between populations of *P. sergenti* is a reflection of differences in the capacity of different populations to transmit *L. tropica* (Depaquit et al. 2002, Baron et al. 2008).

P. (*Phlebotomus*) *papatasi* shares a distribution similar to *P. alexandri* and *P. sergenti*. It is a vector of *Leishmania major*. Microsatellite variation has been identified

between populations of *P. papatasi*, which may prove useful in understanding the movement of the species (Hamarsheh et al. 2009). A number of studies have employed genetic markers to determine the homogeneity of populations of *P. papatasi* across its range. Studies examining the mitochondrial ND4, cyt b, and ITS2 have found little variation across populations of this species (Esseghir et al. 1997, Parvizi et al. 2003, Hamarsheh et al. 2007, Depaquit et al. 2008).

To date, no studies have been published that examine the molecular phylogeny of *P. alexandri* from different populations. While this species is not as well studied as either *P. sergenti* or *P. papatasi*, its large range and status as a vector of visceral leishmaniasis could make this an important species in the epidemiology of leishmaniasis across a very large area. In order to better understand the role of *P. alexandri* in disease transmission, this species must be studied more thoroughly. The purpose of the present study is to examine both cyt b mtDNA and ITS2 of *P. alexandri* from populations in Iraq and Turkey to determine if there are differences between populations.

Methods

This study examined male *P. alexandri* specimens from Iraq and Turkey. The examination of cytochrome b included 21 specimens from six locations. The examination of ITS2 includes 21 specimens from five locations in Iraq. Table 1 gives sample origins and the number of samples from each site.

All specimens included in this analysis were collected using standard CDC light traps. The samples from Turkey were collected in August 2006. The samples from Iraq were collected by U.S. military entomologists as part of the Operation Iraqi Freedom (OIF) Leishmaniasis Control Program from 2003 - 2007. Once collected, the samples used in this study were shipped to the Walter Reed Army Institute of Research (WRAIR), in Silver Spring, Maryland, or the US Army Center for Health Promotion and Preventive Medicine – Europe (CHPPM-E), in Landstuhl, Germany. Male sand flies were preserved in 80% ethyl alcohol.

Sand flies were sorted to subgenera by examination of the male external genitalia using a dissecting microscope. All specimens from the *Paraphlebotomus* subgenus were further identified using a slide mount. The head and male external genitalia were removed from the specimen and placed onto a microscope slide, with polyvinyl acetate (PVA) as the mounting media. The remainder of the body (thorax, abdomen, wings, and legs) were placed into a 1.5 mL microcentrifuge tube and stored at -20^{o}C. Slides were examined to determine species. Once *P. alexandri* specimens were identified, the remainder of the body was used for DNA extraction.

DNA was extracted from the portions of the body not used for identification using the QIAamp DNA Mini Kit (Qiagen) using a modified protocol (Depaquit et al. 2005). Extracted DNA was stored at -20^{o}C. PCR was performed using puReTaq Ready-to-Go PCR Beads (GE Life Sciences). For PCR set-up, 5 μL or template, 0.4 μL each of the forward and reverse primers, and 19.2 μL of water were added to the tube containing the PCR bead. For cyt b, the primers were C3B-PDR and N1N-PDR (Esseghir et al. 1997, Ready et al. 1997). The primers used for ITS2 were C1a and JTS3 (Depaquit et al. 2000). For the cyt b PCR, the initial denaturation was at 94^{o}C for 3 minutes. This was followed by five cycles of denaturation at 94^{o}C for 30 sec, annealing at 40^{o}C for 30 sec, and elongation at 68^{o}C for 1 min. Following this, there were 35 cycles of denaturation at 94^{o}C for 30 sec, annealing at 44^{o}C for 30 sec, and elongation at 68^{o}C for 1 min. The final elongation step was at 68^{o}C for 10 min (Depaquit 2007). For ITS2, the initial denaturation was at 94^{o}C for 3 minutes. This was followed by 40 cycles with denaturation at 94^{o}C for 30 sec, annealing at 50 – 68.5^{o}C for 30 sec, and elongation at 68^{o}C for 1 min. The final elongation step was at 68^{o}C for 10 min (Depaquit 2007).

The cyt b PCR product purified using the QIAquick PCR Purification Kit (Qiagen) and directly sequenced in both directions using the same primers that were used in the PCR reaction. For ITS2, PCR product was run through gel electrophoresis in a 1.5% agarose gel. The band was excised from the gel. DNA was extracted from the gel fragment and purified using the QIAquick Gel Extraction Kit (Qiagen). Sixteen specimens were then directly sequenced using both primers and six samples were cloned using the Invitrogen TOPO TA cloning kit (pCR 2.1 TOPO) (table 1). Sequencing was

performed by the Biomedical Instrumentation Center at the Uniformed Services University of the Health Sciences, Bethesda, Maryland.

Sequence alignment was performed using AlignX, part of the Vector NTI software package (Invitrogen). Phylip version 3.68 was used to perform phylogenetic analysis. The same steps were followed in Phyllip for all data sets. The cyt b and ITS2 sequences (GENBANK numbers AF161215 and AF218316, respectively) for *Phlebotomus (Phlebotomus) duboscqi* were used as outgroups in the analyses. For the ITS2 analysis, the *P. alexandri* ITS2 sequence obtained from Syria (GENBANK number AF218260) was also included in the analysis. The program SEQBOOT was used to perform bootstrap analysis, generating 1000 random datasets from the original data. DNAdist and NEIGHBOR were used to perform neighbor-joining distance analysis. DNA Penny was used to perform maximum parsimony analysis using the branch and bound algorithm. DNAml was used to perform maximum likelihood. The program CONSENSE was used after each analysis to create a consensus tree of the results for each of the three analysis methods.

Results

Cytochrome b

The size of the cyt b sequences obtained from the samples ranged from 421 to 465 bp (table 1). The alignment of the cyt b sequences was 470 bp long. Using Maximum parsimony, the samples from Turkey and Iraq are distinct, with the bootstrap value for the initial branching of each group being 100% (figure 2). Within the set of specimens from Iraq, there is no grouping based on site. The results obtained using the neighbor-joining and maximum-likelihood procedures were similar (figures 3 and 4).

ITS2

The size of sequences obtained for ITS2 varied greatly. For the sequences that were obtained using a cloning procedure, the size range was from 504 to 517 bp. In the direct sequencing procedure, the reverse primer did not work. Due to time and resource constraints, the results reported here for direct sequencing of only the forward strand. For the sequences obtained directly, the sizes ranged from 190 to 444 bp. Two separate alignments and phylogenetic analyses were performed, one that includes only those sequences with more than 400 bp and another that includes those sequences with more than 300 bp.

Eight samples (2 from Turkey, 6 from Iraq) were included in the alignment including only those sequences with more than 400 bps. This alignment was 537 bp long. Ten samples (2 from Turkey, 8 from Iraq) were included in the alignment including those sequences with greater than 300 bp. This alignment was 538 bp long. The maximum parsimony approach showed a separation between the Iraq and Turkey

samples with both sequence alignments, but no clear separation between sites in Iraq (figures 5 and 6). The results using the neighbor-joining and maximum likelihood procedures were similar.

Discussion

This is the first study examining genetic variations among populations of *P. alexandri* across any portion of the species' range. While this species is present across the Mediterranean east to China, it has received relatively little attention from the research community. Guan et al (1986) demonstrated the ability of this species to transmit *L. donovani* in China. Female *P. alexandri* infected with *L. infantum* have been collected from Iran (Azizi et al. 2006). However, *P. alexandri* has not been shown to be capable of transmitting *L. infantum* at this point, so it is not yet a confirmed vector. Given the wide range of *P. alexandri* and the suspected and confirmed vector status, this species should not be neglected.

The cyt b and ITS2 genetic markers were selected to complement each other in this study and provide a clearer picture of any differences between populations. ITS2 is a portion of non-functional RNA located between the 5.8s and 28S rRNA segments (Avise 1994). It has been used in both intraspecific and interspecific population genetics research because it is subject to high rates of change as a result of being a non-coding region (Avise 1994). It has been used in the study of both intra- and inter-specific variation in a variety of arthropod disease vectors, including sand flies (Depaquit et al. 2000, Di Muccio et al. 2000, Depaquit et al. 2002, Depaquit et al. 2008). As part of the mitochondrial DNA, cyt b is maternally inherited and the sequence has a fast rate of evolution (Avise et al. 1987). Cyt b has been used to examine relationships among many groups of organisms, including sand flies (Esseghir et al. 1997, Ready et al. 1997, Moin-Varizi et al. 2007, Baron et al. 2008).

While the analysis presented in this study is not conclusive regarding the status of *P. alexandri* across its range, there does appear to be some population differences between members of this species from Iraq and from the Aegean coast of Turkey. Certainly some amount of difference could be expected between sand flies in central Iraq and those on the Aegean coast of Turkey. Between the two sampling regions lie the Taurus Mountains, which extend across the Southern portion of Turkey along the borders of Syria, Iraq and Iran, and the Syrian Desert, which is west of the sites in Iraq and extends across much of Syria toward the Mediterranean coast. These two major land forms between the sampling sites in the present study could provide effective barriers to mixing of the populations.

In the present study, the Turkey and Iraq specimens grouped separately for both the cyt b and ITS2 analysis. While this may simply be illustrating expected genetic variation over a large geographical area, it is possible that the populations could be different enough that they may actually be more than one species. In order to determine the answer to this question, samples from a broader portion *P. alexandri*'s range must be examined and the ITS2 sequences for the samples in the present study need to be resolved.

The work presented here is part of a larger collaboration with Dr. Jerome Depaquit, University of Reims, to examine this species across as much of its range as possible. Dr. Depaquit is working with specimens from other locations throughout *P. alexandri*'s range, including Iran, Syria, and Spain. Once the ITS2 sequences for the Iraq and Afghanistan samples are determined and the results from the present study are combined with the work being done in Dr. Depaquit's laboratory, a clearer answer to the

question of whether there is real difference between populations should emerge. This, coupled with laboratory based vector incrimination work with *P. alexandri* and *L. infantum*, could help more clearly establish the relative importance of *P. alexandri* in public health.

Table 1. Origin of *Phlebotomus alexandri* specimens, length of the cyt b and ITS2 fragments, and method for sequencing ITS2.

Country	Location	Sample #	Cyt b # base pairs	ITS2 # base pairs	ITS2 method
Iraq	Balad	IZ60	---	517	Cloning
		IZ63	432	514	Cloning
		IZ64	460	---	Cloning
		IZ65	436	---	Cloning
	B'quba	IZ29	451	377	Direct
		IZ30	442	308	Direct
		IZ31	445	368	Direct
		IZ33	441	374	Direct
	Taji	IZ72	421	190	Direct
		IZ73	421	440	Direct
		IZ74	450	296	Direct
		IZ75	453	373	Direct
		IZ76	---	220	Direct
	Tallil	IZ51	438	---	Cloning
		IZ52	439	504	Cloning
		IZ53	436	514	Cloning
		IZ55	431	---	Direct
		IZ56	---	516	Cloning
	Tikrit	IZ69	449	---	Direct
		IZ70	455	---	Direct
		IZ71	460	214	Direct
Turkey	Akbuk	TK07	464	443	Direct
		TK09	446	444	Direct
		TK17	465	225	Direct

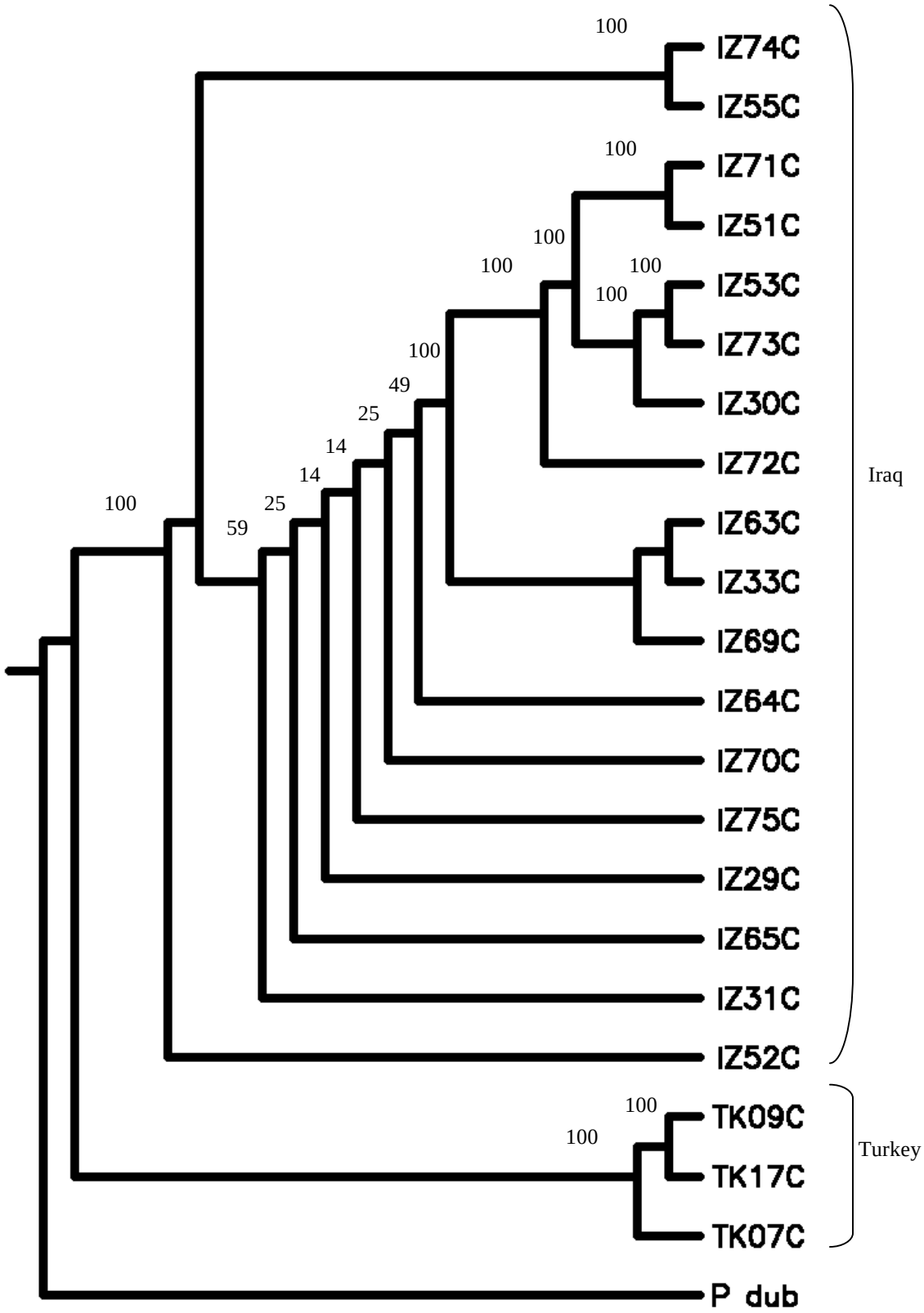

Figure 1. Phylogenetic tree for cytochrome b developed using maximum parsimony analysis. Percentages are derived from the bootstrap analysis.

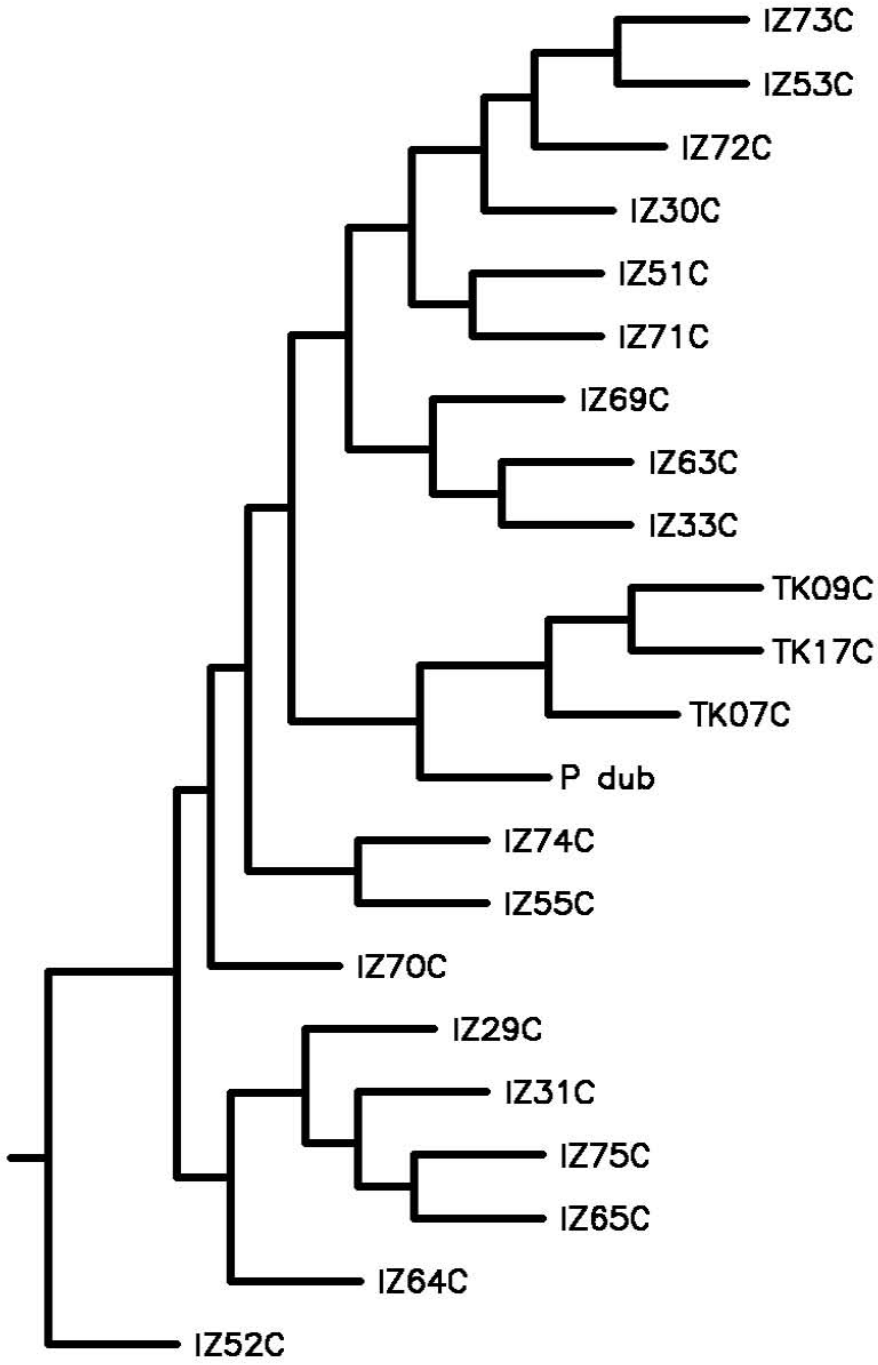

Figure 2. Phylogenetic tree developed for cytochrome b using neighbor-joining distance analysis.

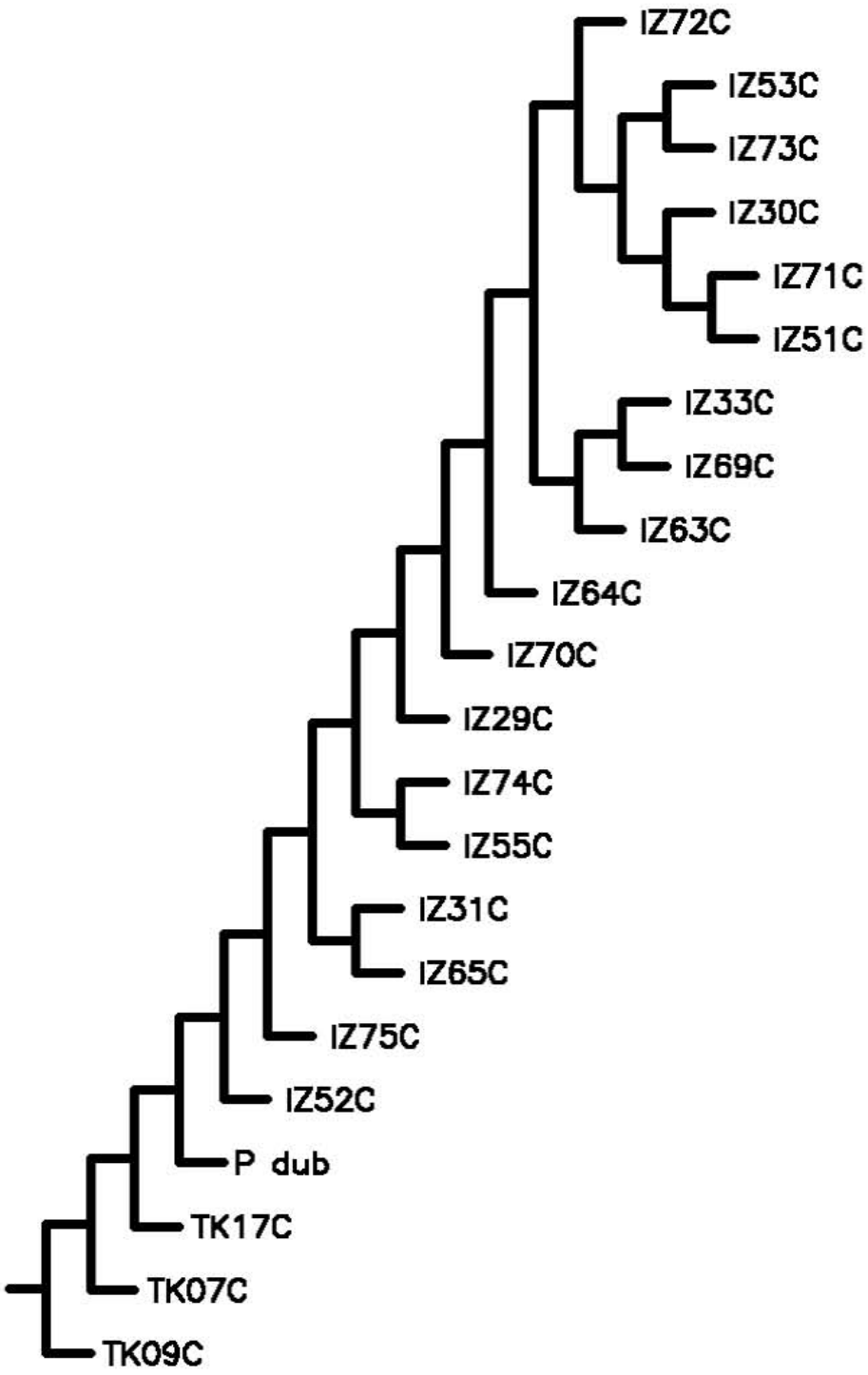

Figure 3. Phylogenetic tree for cytochrome b developed using maximum likelihood analysis.

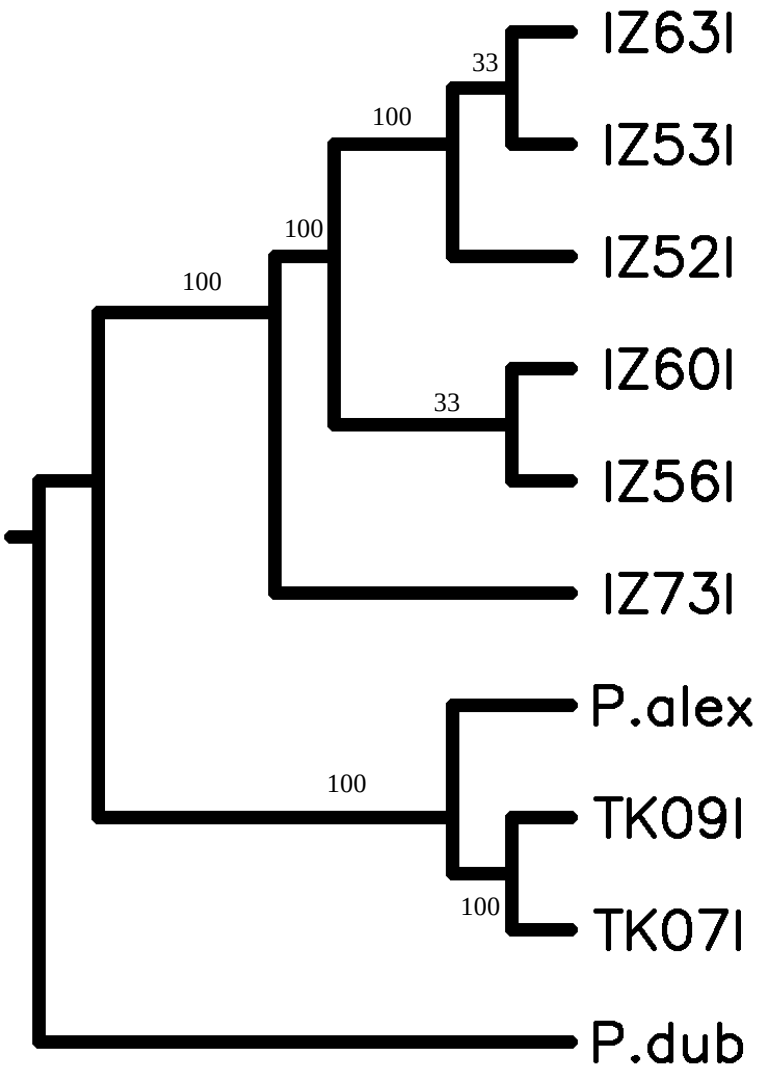

Figure 4. Phylogenetic tree for ITS2 samples with more than 400 base pairs using the maximum parsimony approach.

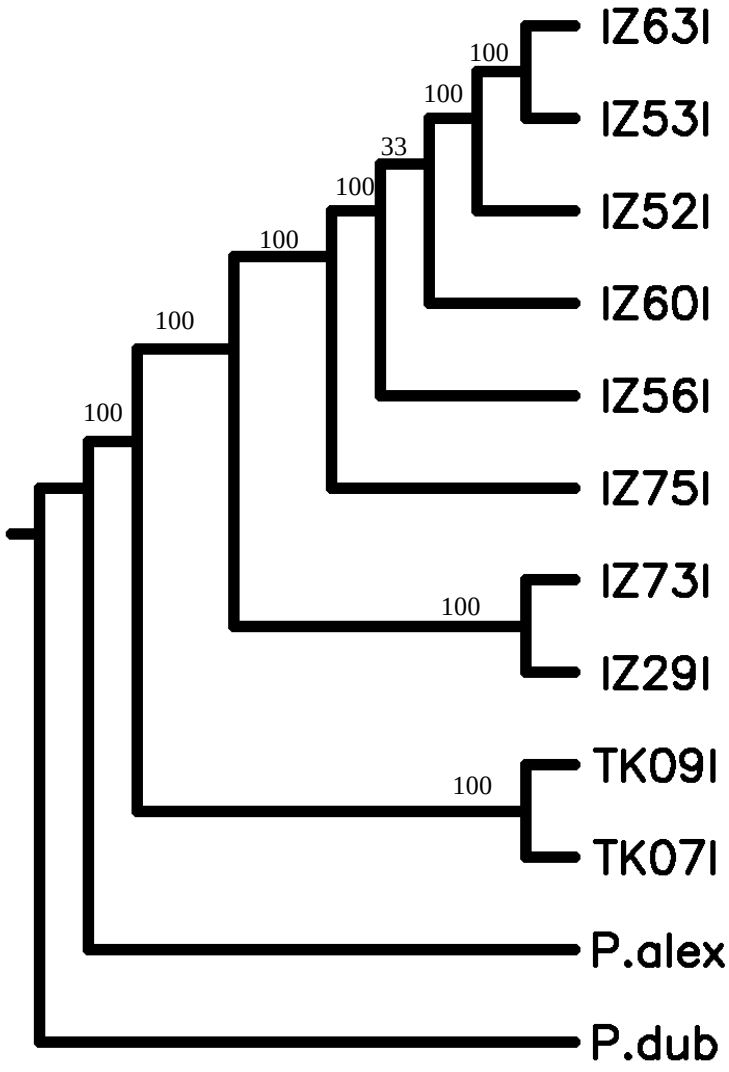

Figure 5. Phylogenetic tree for ITS2 samples with more than 300 base pairs using the maximum parsimony approach.

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Chapter 6

Conclusion

The seeds of an idea for this research started when I was deployed as the Force Health Protection Officer for Combined Joint Task Force Seven (CJTF-7) during the first year of Operation Iraqi Freedom. Seeing the somewhat disconnected and reactionary response to the emerging leishmaniasis problem among the U.S. military units in Iraq, I became interested in the problems surrounding leishmaniasis and sand flies, with a particular focus on how and why the vectors interact with their environment, hosts, and the pathogens that they transmit.

The research presented in this dissertation was designed fundamentally to try and contribute to the body of knowledge surrounding sand fly biology and ecology in the Middle East. In the first three sections of this dissertation, different relationships between sand flies and their environment were explored on a variety of scales in an attempt to identify patterns and relationships that may enhance the understanding of the biology of the flies and the epidemiology of the diseases they transmit. The fourth section of this research is a bit removed from the first three in that the theme of ecology is abandoned for an examination of the population genetics of *P. alexandri*. While this may seem disconnected from the rest of the research, the question of whether or not there is genetic variation between populations of this species could, ultimately, have an impact on the ecology, biology, and vectoral capacity of this species. Since it has not been previously examined, I had easy access to samples from several locations and a collaborator with a pool of specimens from different locations, it was logical to include that examination in a dissertation exploring the biology and ecology of sand flies in the Middle East region.

In the first study reported here, temperature, wind speed, and sky cover had significant affects on the sand fly activity at TAB. Therefore, it was concluded that the sand fly activity would be highest on warm, clear nights, with low wind speed. Activity was highest when the mean night time temperature was above 30^{o}C and below 24^{o}C. While it was impossible to determine the affect of the independent variables on specific species of sand flies, the trends identified here may help shed some light on how and when to conduct surveillance and control efforts for sand flies. Additionally, the strong inverse relationship between moon illumination and sand flies collected provides evidence that reliance solely on the use of unbaited CDC light traps for sand fly surveillance may not be the most effective approach. Civilian public health programs and military preventive medicine units should employ a more comprehensive strategy where possible in order to provide more effective disease surveillance. In order to make that possible, research much continue to examine new and modified surveillance equipment and methods.

The second study focused on the species *P. papatasi* and *P. alexandri*, the two species which were consistently the most abundant in the collections from Iraq and both important vector species. The aim of this study was to develop niche models of the distribution of these two species using remotely sensed land cover, temperature, and precipitation variables. While the temperature and precipitation variables contributed to the development of the model, land cover drove model development. The models created in this study may be used to help shape surveillance strategies and guide vector and disease control programs. In the future, I plan to expand the scope of these models to include the full range of the species. I also hope to expand my niche modeling efforts to

include other sand fly vectors. This is a powerful tool that has the potential to help improve the understanding of the ecoepidemiology of leishmaniasis. These tools may be adapted to benefit regional disease prevention and control programs as well help shape military medical threat assessments.

An early version of the niche modeling project included NDVI data as one of the layers in the project. However, NDVI did not contribute appreciably to the model and the model was not adversely affected when it was removed (Appendix A). Therefore, the final model development proceeded without the inclusion of NDVI data. The failure of NDVI to contribute to the niche model led to the ultimate design of the third study in this work. In order to address the question of whether or not NDVI shows any relationship to sand fly population or activity levels, I used sand fly collection data from Iraq along with NDVI at a 250 m resolution. The niche modeling project attempted to use 1 km resolution NDVI data since the modeling area was so large. I hypothesized that the 250 m resolution data might be a better starting point given the short distances that sand flies travel and the weak nature of sand fly flight.

While some significant correlations were identified between NDVI and *P. papatasi* collections, the study presented here only looked at one year's worth of sand fly collection and NDVI data. In the future, I plan to expand this study beyond the present scope by:

1. Obtaining the same AVHRR imagery used by Cross et al (1996) in order to directly compare the NDVI values between the two studies and help broaden the examination of the relationships between sand fly activity and NDVI.

2. Incorporating multiple-year NDVI layers and averaging them across months to create a monthly average of NDVI values to avoid any potential anomaly from just using one year's worth of imagery.
3. Incorporating the 2003 and/or 2004 field collection data into this study in order to increase the number of sites under examination. The 2005 collection year was originally chosen because it is a more uniform data set than either then 2003 and 2004 collection years. However, the collection records can be combined and used in the analysis with the multiple-year NDVI data.

Since NDVI has been successfully applied to predict population levels of other disease vectors and outbreaks of Rift Valley Fever, there is a great deal of interest in determining the applicability of this index to sand flies. While I do not expect this further analysis to definitively answer the question of whether there is a relationship between NDVI and *P. papatasi* population levels, I think that pursuing these expanded areas of research will help get closer to the answer.

In the final section of this research, I turned away from the ecological work, more closely examining *P. alexandri* for genetic differences between populations. While there does appear to be some distinction between the Turkey and Iraq populations, the geographic area covered here only represents a small portion of the range of these species. The work presented here is part of a larger collaborative effort with Dr. Jerome Depaquit, University of Reims, France. Once the work the full collaborative effort is complete, we should have a good representation of *P. alexandri* from across its range and be able to explore the question of genetic difference more thoroughly. If there are significant genetic differences across populations of *P. alexandri*, this could impact the

understanding and future direction of research into the biology, ecology, and vectorial capacity of this species. If there are not significant genetic differences between populations, there still needs to be more investigation into all aspects of the biology of this species.

The primary goal for future *P. alexandri* research needs to be in determining whether or not this species is a competent vector of *L. infantum*. This would require colonization of the fly in the laboratory environment and subsequent vector incrimination studies. If *P. alexandri* were proven to be a competent vector of *L. infantum* along with *L. donovani*, it would have interesting implications on the epidemiology of VL in the Old World. There would essentially be one competent vector of both Old World forms of VL whose range corresponds to the entire range of *L. infantum* and much of the range of *L. donovani*. While *P. alexandri* may not be the primary vector across its entire range, its presence in areas with zoonotic *L. infantum* could help maintain the cycle of disease in the environment. The vector potential of this species is one of the reasons that it is important to answer the question of whether or not this is one species across its range or a species complex. If this species were to actually be a species complex, the ecology, biology, and behavior of different populations would need to be investigated more closely to determine the real role of *P. alexandri* in the epidemiology of leishmaniasis.

The work presented here attempted to make some inroads into the overall understanding of the biology and ecology of the sand flies in the Middle East with a focus on the two most common vector species collected during Operation Iraqi Freedom. In the course of pursuing this research, I developed far more questions than answers. Each part of this research adds to the body of knowledge of some aspect of sand fly

biology and ecology. However, there is much left to be discovered, particularly in the case of *P. alexandri* and other lesser known sand fly species.

Appendix A

Phlebotomus alexandri cytochrome b sequence alignment

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22              470
IZ51C      ?????????? ?TCGTTCTAT TCCTAATAAA TTAGGAGG-A GTAATTGCCC
IZ71C      ?????????? TTCGTTCTAT TCCTAATAAA TTAGGAGGGA GTAATTGCCC
IZ53C      ?????????? -TCGTTCTAT TCCTAATAAA TTAGGGGG-A GTAATTGCCC
IZ73C      ?????????? ?????????? ???????AAA TTAGGGGG-A GTAATTGCCC
IZ72C      ?????????? ?????????? ???????AAA TTAGGGGG-A GTAATTGCCC
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IZ63C      ?????????? ???????TAT TCCTAATAAA TTAGGGGG-A GTAATTGCCC
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IZ69C      ?????????? ??CGTTCTAT TCCTAATAAA TTAGGGGG-A GTAATTGCCC
P_dub      ?????????? ?????????? ?CCAAATAAA TTAGGAGG-A GTAATTGCTC
TK07C      TATGCTATTC TTCGTTCAAT TCCCAATAAA TTAGGAGG-A GTAATTGCTC
TK09C      ???????TTC TTCGTTCAAT TCCCAATAAA TTAGGAGG-A GTAATTGCTC
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IZ55C      ?????????? ?????TCTAT TCCTAATAAA TTAGGGGG-A GTAATTGCCC
IZ74C      ?????????? TTCGTTCTAT TCCTAATAAA TTAGGGGG-A GTAATTGCCC
IZ70C      ?????????C TTCGATCTAT TCCTAATAAA TTAGGGGG-A GTAATTGCCC
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IZ64C      ????????TC TTCGTTCTAT TCCTAATAAA TTAGGGGG-A GTAATTGCCC
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IZ65C      ?????????? ???GTTCTAT TCCTAATAAA TTAGGGGG-A GTAATTGCCC
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TATAGTAATT ACAATTATTC TCCTAACATG AATTGGAGCT CGTCCTGTTG
TATAGTAATT ACAATTATTC TCCTAACATG AATTGGAGCT CGTCCTGTTG
TATAGTAATT ACAATTATTC TCCTAACATG AATTGGAGCT CGTCCTGTTG
TATAGTAATT ACAATTATTC TCCTAACATG AATTGGAGCT CGTCCTGTTG

AAACACCTTA TATTTTAACA GGTCAAATCT TAACAGTCTT ATATTTCTCT
AAACACCTTA TATTTTAACA GGTCAAATCT TAACAGTTTT ATATTTCTCT
AAACACCTTA TATTTTAACT GGTCAAATCT TAACAGTCTT ATACTTCTCT
AAACACCTTA TATTTTAACA GGTCAAATCT TAACAGTCTT ATATTTCTCT
AAACACCTTA TATTTTAACA GGTCAAATCT TAACAGTCTT ATATTTCTCT
AAACACCTTA TATTTTAACA GGTCAAATCT TAACAGTCTT ATATTTCTCT
AAACACCTTA TATTTTAACA GGTCAAATCT TAACAGTTTT ATATTTCTCT
AAACACCTTA TATTTTAACA GGTCAAATCT TAACAATTTT ATATTTCTCT
AAACACCTTA TATTTTAACA GGTCAAATCT TAACAGTTTT ATATTTTTCT
AAACCCCTTA CATTTTAACA GGCCAAATTT TAACAATTCT TTATTTTTCA
AAACTCCTTA TATTTTAACT GGTCAAATTT TAACTGTTTT ATATTTTTCT
AAACTCCTTA TATTTTAACT GGTCAAATTT TAACTGTTTT ATATTTTTCT
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AAACTCCTTA TATTTTAACT GGTCAAATTT TAACTGTTTT ATATTTTTCT
AAACCCCTTA TATTTTAACA GGTCAAATCT TAACAGTCTT ATATTTCTCT
AAACCCCTTA TATTTTAACA GGTCAAATCT TAACAGTCTT ATATTTCTCT
AAACTCCTTA TATTTTAACA GGTCAAATCT TAACAGTCTT ATATTTCTCT
AAACTCCTTA TATTTTAACA GGTCAAATCT TAACAGTCTT ATATTTCTCT
AAACTCCTTA TATTTTAACA GGTCAAATCT TAACAGTCTT ATATTTCTCT
AAACTCCTTA TATTTTAACA GGTCAAATCT TAACAGTCTT ATATTTCTCT
AAACTCCTTA TATTTTAACA GGTCAAATCT TAACAGTCTT ATATTTCTCT
AAACTCCTTA TATTTTAACA GGTCAAATCT TAACAGTCTT ATATTTCTCT
AAACTCCTTA TATTTTAACA GGTCAAATCT TAACAGTCTT ATATTTCTCT

TACTATATTT TAAACCCAAT TATCTCAAAA CTATGAGACA ATCTATTAAA
TACTATATTT TAAACCCAAT TATCTCAAAA CTATGAGACA ATCTATTAAA
TACTATATTT TAAACCCAAT TATCTCAAAA CTATGAGACA ATCTATTAAA
TACTATATTT TAAACCCAAT TATCTCAAAA CTATGAGACA ATCTATTAAA
TACTATATTT TAAACCCAAT TATCTCAAAA CTATGAGACA ATCTATTAAA
TACTATATTT TAAACCCAAT TATCTCAAAA CTATGAGACA ATCTATTAAA
TACTATATTT TAAACCCAAT TATCTCAAAA CTATGAGACA ATCTATTAAA
TACTATATTT TAAACCCAAT TATCTCAAAA CTATGAGACA ATCTATTAAA
TACTATATTT TAAACCCAAT TATCTCAAAA CTATGAGACA ATCTATTAAA
TACTTTATCT TAAATCCAAT TACCTCAAAA TTATGAGATA ATCTACTCAA
TATTATATTT TAAATCCTAT TATCTCTAAA CTATGAGATA ATTTATTAAA
TATTATATTT TAAATCCTAT TATCTCTAAA CTATGAGATA ATTTATTAAA
TATTATATTT TAAATCCTAT TATCTCTAAA CTATGAGATA ATTTATTAAA
TACTATATTT TAAACCCAAT CATCTCAAAA CTATGAGACA ATCTATTAAA
TACTATATTT TAAACCCAGT CATCTCAAAA CTATGAGATA ATCTATTAAA
TATTATATTT TAAACCCAAT TATCTCAAAA CTATGAGACA ATCTATTAAA
TACTATATTT TAAACCTAAT TATCTCAAAA CTATGAGACA ATCTATTAAA
TACTATATTT TAAACCCAAT TATCTCAAAA CTATGAGACA ATCTATTAAA
TACTATATTT TAAACCCAAT TATCTCAAAA CTATGAGACA ATCTATTAAA
TACTATATTT TAAACCCAAT TATCTCAAAA CTATGAGACA ATCTATTAAA
TACTATATTT TAAACCCAAT TATCTCAAAA CTATGAGACA ATCTATTAAA
TACTATATTT TAAACCCAAT TATCTCAAAA CTATGAGACA ATCTATTAAA

TAATTAGTTA ATGAGCTTGA ATAGCAATTG TTTTGAAAAC ATTAGATAGA
TAATTAGTTA ATGAGCTTGA ATAGCAATTG TTTTGAAAAC ATTAGATAGA
TAATTAGTTA ATGAGCTTGA ATAGCAATTG TTTTGAAAAC ATTAGATAGA
TAATTAGTTA ATGAGCTTGA ATAGCAATTG TTTTGAAAAC ATTAGATAGA
TAATTAGTTA ATGAGCTTGA ATAGCAATTG TTTTGAAAAC ATTAGATAGA
TAATTAGTTA ATGAGCTTGA ATAGCAATTG TTTTGAAAAC ATTAGATAGA
TAATTAGTTA ATGAGCTTGA ATAGCAATTG TTTTGAAAAC ATTAGATAGA
TAATTAGTTA ATGAGCTTGA ATAGCAATTG TTTTGAAAAC ATTAGATAGA
TAATTAGTTA ATGAGCTTGA ATAGCAATTG TTTTGAAAAC ATTAGATAGA
TAATTAGTTA ATAAGCTTTA ATAGCAATTG TTTTGAAAAC ATTAGATAGA
TAATTAGTTA ATGAGCTTGA ATAGCAATTG TTTTGAAAAC ATTAGATAGA
TAATTAGTTA ATGAGCTTGA ATAGCAATTG TTTTGAAAAC ATTAGATAGA
TAATTAGTTA ATGAGCTTGA ATAGCAATTG TTTTGAAAAC ATTAGATAGA
TAATTAGTTA ATGAGCTTGA ATAGCAATTG TTTTGAAAAC ATTAGATAGA
TAATTAGTTA ATGAGCTTGA ATAGCAATTG TTTTGAAAAC ATTAGATAGA
TAATTAGTTA ATGAGCTTGA ATAGCAATTG TTTTGAAAAC ATTAGATAGA
TAATTAGTTA ATGAGCTTGA ATAGCAATTG TTTTGAAAAC ATTAGATAGA
TAATTAGTTA ATGAGCTTGA ATAGCAATTG TTTTGAAAAC ATTAGATAGA
TAATTAGTTA ATGAGCTTGA ATAGCAATTG TTTTGAAAAC ATTAGATAGA
TAATTAGTTA ATGAGCTTGA ATAGCAATTG TTTTGAAAAC ATTAGATAGA
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TAATTAGTTA ATGAGCTTGA ATAGCAATTG TTTTGAAAAC ATTAGATAGA
TAATTAGTTA ATGAGCTTGA ATAGCAATTG TTTTGAAAAC ATTAGATAGA

AACTAAAATT TTCTATTAAC TTTACTAAAA TTAATTATTA TAATATAATT
AACTAAAATT TTCTATTAAC TTTACTAAAA TTAATTATTA TAATATAATT
AACTAAAATT TTCTATTAAC TTTACTAAAA TTAATTATTA TAATATAATT
AACTAAAATT TTCTATTAAC TTTACTAAAA TTAATTATTA TAATATAATT
AACTAAAATT TTCTATTAAC TTTACTAAAA TTAATTATTA TAATATAATT
AACTAAAATT TTCTATTAAC TTTACTAAAA TTAATTATTA TAATATAATT
AACTAAAATT TTCTATTAAC TTTACTAAAA TTAATTATTA TAATATAATT
AACTAAAATT TTCTATTAAC TTTACTAAAA TTAATTATTA TAATATAATT
AACTAAAATT TTCTATTAAC TTTACTAAAA TTAATTATTA TAATATAATT
AATTAAAATT TTCTATTAAC TTTACTATTT TTAATTATTA TAATAATATT
AACTAAAATT TTCTATTAAC TTTACTAAAT TTAATTATTA TAATATAATT
AACTAAAATT TTCTATTAAC TTTACTAAAT TTAATTATTA TAATATAATT
AACTAAAATT TTCTATTAAC TTTACTAAAT TTAATTATTA TAATATAATT
AACTAAAATT TTCTATTAAC TTTACTAAAA TTAATTATTA TAATATAATT
AACTAAAATT TTCTATTAAC TTTACTAAAA TTAATTATTA TAATATAATT
AACTAAAATT TTCTATTAAC TTTACTAAAA TTAATTATTA TAATATAATT
AACTAAAATT TTCTATTAAC TTTACTAAAA TTAATTATTA TAATATAATT
AACTAAAATT TTCTATTAAC TTTACTAAAA TTAATTATTA TAATATAATT
AACTAAAATT TTCTATTAAC TTTACTAAAA TTAATTATTA TAATATAATT
AACTAAAATT TTCTATTAAC TTTACTAAAA TTAATTATTA TAATATAATT
AACTAAAATT TTCTATTAAC TTTACTAAAA TTAATTATTA TAATATAATT

ATCCTTAATC CAATAAATAA ACAAATAAAA CATAAAGAAG CAGGTAAATA
ATCCTTAATC CAATAAATAA ACAAATAAAA CATAAAGAAG CAGGTAAATA
ATCCTTAATC CAATAAATAA ACAAATAAAA CATAAAGAAG CAGGTAAA??
ATCCTTAATC CAATAAATAA ACAAATAAAA CATAAAGAAG CAGGTAAAT?
ATCCTTAATC CAATAAATAA ACAAATAAAA CATAAAGAAG CAGGTAAAT?
ATCCTTAATC CAATAAATAA ACAAATAAAA CATAAAGAAG CAGGTAAAT?
ATCCTTAATC CAATAAATAA ACAAATAAAA CATAAAGAAG CAGGTAAATA
ATCCTTAATC CAATAAATAA ACAAATAAAA CATAAAGAAG CAGGTAAAT?
ATCCTTAATC CAATAAATAA ACAAATAAAA CATAAAGAAG CAGGTAAATA
AATTTTAATC CAATAAATAA ACAAATAAAA CATAAAGAAG CAGGTAAATA
ATTTTTAAAC CAACAAATAA ACAAATAAAA CATAACGAAG CTGGTAAATA
ATTTTTAAAC CAACAAATAA ACAAATAAAA CATAACGAAG CTGGTAAATA
ATTTTTAAAC CAACAAATAA ACAAATAAAA CATAACGAAG CTGGTAAATA
ATCCTTAATC CAATAAATAA ACAAATAAAA CATAAAGAAG CAGGTAA???
ATCCTTAATC CAATAAATAA ACAAATAAAA CATAAAGAAG CAGGTAAATA
ATCCTTAATC CAATAAATAA ACAAATAAAA CATAAAGAAG CAGGTAAATA
ATCCTTAATC CAATAAATAA ACAAATAAAA CATAAAGAAG CAGGTAAATA
ATCCTTAATC CAATAAATAA ACAAATAAAA CATAAAGAAG CAGGTAAATA
ATCCTTAATC CAATAAATAA ACAAATAAAA CATAAAGAAG CAGGTAAAT?
ATCCTTAATC CAATAAATAA ACAAATAAAA CATAAAGAAG CAGGTAAATA
ATCCTTAATC CAATAAATAA ACAAATAAAA CATAAAGAAG CAGGTAAATA
ATCCTTAATC CAATAAATAA ACAAATAAAA CATAAAGAAG CAGGTAAATA

?????????? ??????????
TCTCTTTCAT ACTAAATACA
?????????? ??????????
?????????? ??????????
?????????? ??????????

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?????????? ??????????
?????????? ??????????
?????????? ??????????
TCTCTTTCAT AC????????
ACTCTT???? ??????????
TCTTTTTCAT ACTAA?????
TCTT?????? ??????????
TCTTTTTCAT ACTAAATAT?
?????????? ??????????
TCTCTTTCAT A?????????
TCTCTTTCAT ACTAA?????
?????????? ??????????
TCTCTTTCAT ACTAAATAC?
?????????? ??????????
?????????? ??????????
TCTCTTTCAT ACTAA?????
TCTCTTT??? ??????????
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Appendix B

***Phlebotomus alexandri* ITS2 sequence alignment:**

Sequences with more than 400 base pairs

```
10         537
IZ53I      AATTCGCCCT TCGCAGCTAA CTGTGTGAAA TCGTGTGAAC TGCAGGACAC
IZ63I      AATTCGCCCT TCGCAGCTAA CTGTGTGAAA TCGTGTGAAC TGCAGGACAC
IZ56I      AATTCGCCCT TCGCAGCTAA CTGTGTGAAA TCGTGTGAAC TGCAGGACAC
P.alex     ?????????? ??CGCAGCTA ACTGTGTGAA ATCGTGTGAA CTGCAGGACA
TK07I      ?????????? ?????????? ?????????? ?????????? ??????????
TK09I      ?????????? ?????????? ?????????? ?????????? ??????????
IZ73I      ?????????? ?????????? ?????????? ?????????G GCTGCGGGAC
IZ60I      AATTCGCCCT TCGCAGCTAA CTGTGTGAAA TCGTGTGAAC TGCAGGACAC
IZ52I      ?????GCCCT TCGCAGCTAA CTGTGTGAAA TCGTGTGAAC TGCAGGACAC
P.dub      ?????????? ??CGCAGCTA ACTGTGTGAA ATCGTGTGAA CTGCAGGACA

ATGAACATCG ACATTTT-GA ACGCATATTG CGGTCCATGC AAAAGTTTAA
ATGAACATCG ACATTTT-GA ACGCATATTG CGGTCCATGC AAAAGTTTAA
ATGAACATCG ACATTTT-GA ACGCATATTG CGGTCCATGC AAAAGTTTAA
CATGAACATC GACATTTTGA ACGCATATTG CGGTCCATGC AAAAGTTTAA
------CTCG AC-TTTT-GA ACGCATATTG CGGTCCATGC AAAAGTTTAA
------CTCG ACATTTT-GA ACGCATATTG CGGTCCATGC AAAAGTTTAA
CTGAAC-TCG AC-TTTT-GA ACGCATATTG CGGTCCATGC AAAAGTTTAA
ATGAACATCG ACATTTT-GA ACGCATATTG CGGTCCATGC AAAAGTTTAA
ATGAACATCG ACATTTT-GA ACGCATATTG CGGTCCATGC AAAAGTTTAA
CATGAACATC GACATTTTGA ACGCATATTG CGGTCCATGC AAAAGTTTAA

ACTTGTTT-- AAACTGCATG GACCACGTAT GGTTGAGTAT CGTAAATATT
ACTTGTTT-- AAACTGCATG GACCACGTAT GGTTGAGTAT CGTAAATATT
ACTTGTTT-- AAACTGCATG GACCACGTAT GGTTGAGTAT CGTAAATATT
ACTTGTTT-- AAACTGCATG GACCACGTAT GGTTGAGTAT CGTAAATATT
ACTTGTTT-- AAACTGCATG GACCACGTAT GGTTGAGTAT CGTAAATATT
ACTTGTTT-- AAACTGCATG GACCACGTAT GGTTGAGTAT CGTAAATATT
ACTTGTTT-- AAACTGCATG GACCACGTAT GGTTGAGTAT CGTAAATATT
ACTTGTTT-- AAACTGCATG GACCACGTAT GGTTGAGTAT CGTAAATATT
ACTTGTTT-- AAACTGCATG GACCACGTAT GGTTGAGTAT CGTAAATATT
ATTTTTTTTT AAACTGCATG GACCACGTAT GGTTGAGTGT CGTAAATATT

AAGCAATTGA AATTGTTTTC TTTTT----- ----A-ATGA ACATTGGAGC
AAGCAATTGA AATTGTTTTC TTTTT----- ----A-ATGA ACATTGGAGC
AAGCAATTGA AATTGTTTTC TTTTT----- ----A-ATGA ACATTGGAGC
AAGCAATTGA AATTGTTTTT TTATTTTTTT TTCTAAAAAA ACATTGGAGC
AAGCAATTGA AATTGTTTTT TATTTTTTTT TTATAAAAAA ACATTGGAGC
AAGCAATTGA AATTGTTTTT TATTTTTTTT TTATAAAAAA ACATTGGAGC
AAGCAATTGA AATTGTTTTC TTTTT----- ----A-ATGA ACATTGGAGC
AAGCAATTGA AATTGTTTTC TTTTT----- ----A-ATGA ACATTGGAGC
AAGCAATTGA AATTGTTTTC TTTTT----- ----A-ATGA ACATTGGAGC
AAGCAATTGA AATTGTTTTC TTTTGT---- ------AA-A ACATTGGAGC

TATGGAAAAT AATTTTTTCA TGCTCTTAAT ATA--TTGTT TTAAAGCATA
TATGGAAAAT AATTTTTTCA TGCTCTTAAT ATA--TTGTT TTAAAGCATA
TATGGAAAAT AATTTTTTCA TGCTCTTAAT ATA--TTGTT TTAAAGCATA
TATGGAAATT AATTTTTTCA TGCTCTTAAT ATATATATTT TTAAAGCATA
TATGGAAATT AATTTTTTCA TGCTCTTAAT ATATATATTT TTAAAGCATA
TATGGAAATT AATTTTTTCA TGCTCTTAAT ATATATATTT TTAAAGCATA
TATGGAAAAT AATTTTTTCA TGCTCTTAAT ATA--TTGTT TTAAAGCATA
TATGGAAAAT AATTTTTTCA TGCTCTTAAT ATA--TTGTT TTAAAGCATA
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TATGGAAAAT AATTTTTTCA TGCTCTTAAT ATA--TTGTT TTAAAGCATA
TATGGAATAA GTATTTTTCA TGCTCTTAAT ATA--TATAT TTTAAGCACA

TTTGAATGTA CCCGATATAT AAATATTAAA AAAAAAATAA -TATAATAAG
TTTGAATGTA CCCGATATAT AAATATTAAA AAAAAAATAA -TATAATAAG
TTTGAATGTA CCCGATATAT AAATATTAAA AAAAAAATAA -TATAATAAG
TTTGAATGTA CCCGATATAT AAATATTAAA AA----AAAT --A---TAAG
TTTGAATGTA CCCGATATAT AAATATTAAA AAAAATATAA ---------G
TTTGAATGTA CCCGATATAT AAATATTAAA AAAAATATAA ---------G
TTTGAATGTA CCCGATATAT AAATATTAAA AAAAAAAAAA ATATAAAAGG
TTTGAATGTA CCCGATATAT AAATATTAAA AAAAAAATAA -TATAATAAG
TTTGAATGTA CCCGATATAT AAATATTAAA AAAAAAATAA -TATAATAAG
TTTGAATGTA CCCAATATAT AA-TATTAAA AAGATGAAAT ----A-TAAG

TGGTATATCA AAGTCAT-GG ATAATTTTTT --CTATAAAA ATTTGAAAAA
TGGTATATCA AAGTCATTGG ATAATTTTTT --CTATAAAA ATTTGAAAAA
TGGTATATCA AAGTCATTGG ATAATTTTTT --CTATAAAA ATTTGAAAAA
TGGTATATCA AAGTCATTGG ATAATTTTTT T-CTATAAAA ATTTGAAAAA
GGGTATATCA AAGTCATTGG ATAATTTTTT TTCTATAAAA ATTTGAAAAA
GGGTATATCA AAGTCATTGG ATAATTTTTT TTCTATAAAA ATTTGAAAAA
GGGTATATCA AAGCCTTTGG AAAATTTTTT --CTATAAAA TTTTGAAAAA
TGGTATATCA AAGTCATTGG ATAATTTTTT --CTATAAAA ATTTGAAAAA
TGGTATATCA AAGTCATTGG ATAATTTTTT --CTATAAAA ATTTGAAAAA
TGGTATATCA TAGTCATTGA ATGATTT--- ------AAAA ATCTTAAA--

GACATACTGT TATACATAAA AGCTTATTTA TTTATTTTTT TTTTTAATAT
GACATACTGT TATACATAAA AGCTTATTTA TTTATTTTTT TTTT-AATAT
GACATACTGT TATACATAAA AGCTTATTTA TTTATTTTTT TTTA--ATAT
GACATATTGT TATACAGAAA AGCTTAT--- -TTTTTTTT- -----AATAT
AACATATTGT TATACAAAAA AGCTTATTTT TT--TTTT-- ------AAAA
AACATATTGT TATACAAAAA AGCTTATTTT TT--TTTA-- ------AAAA
AACTTCCGGT TATACATAAA ACCTTATTTT TTTTTTTTTT TTTT-AAAAA
GACATACTGT TATACATAAA AGCTTATTTA TTTATTTTTT TTTT-AATAT
GACATACTGT TATACATAAA AGCTTATTTA TTTATTTTTT TTTT-AATAT
GACATATTGT TATACAGAAA AACTTATATC TTTTTTTTT- ------AATA

AAAAGGGATT ATT-CACTAT AAAAAATATG CTAAAGAAAA AAAAAA---T
AAAAGGGATT ATT-CACTAT AAAAAATATG CTAAAGAAAA AAAAAA---T
AAAAGGGATT ATT-CACTAT AAAAAATATT CTAAAGAAAA AAAAAAAAAT
AAAAGGGATT ATT-CACTTT AAAAAATATG CACAAAAAAA AACTTA--AT
AAAAGGGGTT TTTTCCTTTT AAAAAATTGG CCCAAAAAAA AATTTA---T
AAAAGGGAAT TATCCCTTTA AAAAAAAAGG CCAAAAAAAA ACTTTA---T
AAAAGGGATT TTT-CCCTAT AAAAAATTTG CTAAA-AAAA AAAAAAAAAT
AAAAGGGATT ATT-CACTAT AAAAAATATG CTAAAGAAAA AAAAAAAAAT
AAAAGGGATT ATT-CACTAT AAAAAATATG CTAAA-GAAA AAAAAAAA-T
AAAAGGGATT ATT-CAATAT ATATAATGTG CAAAAATATA AAATTAT-AT

TGCGATCTCA ACTCATACGT GACTACCCCC TGAATTTAAG CATATTAATA
TGCGATCTCA ACTCATACGT GACTACCCCC TGAATTTAAG CATATTAATA
TGCGATCTCA ACTCATACGT GACTACCCCC TGAATTTAAG CATATTAATA
TGCGATCTCA ACTCATACGT GACTACCCCC TGAATTTAAG CATATTAATA
TGGGAATCCC ACCCCAAACG GGCCCCCCCC CGGATTTTAA CCATTTTAAA
TGGGGTCCCC ACCCCAAAGG GGCCCCCCCC CGGATTTAAA GCTTTTTAAA
TGGGGTTCCC ACCCCAAAGG GACACCCCCC CGAATTTATA CCTTTTAAAA
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TGCGATCTCA ACTCATACGT GACTACCCCC TGAATTTAAG CATATTAATA
TGCGATCTCA ACTCATACGT GACTACCCCC TGAATTTAAG CATATTAATA
TGCGATCTCA ACTCATACGT GACTACCCCC TGAATTTAAG CATATTAATA

AGCGGAGGAA AAGAAACTAA CCAGGAAGGG CGAATTC
AGCGGAGGAA AAGAAACTAA CCAGGAAGGG CGAATTC
AGCGGAGGAA AAGAAACTAA CCAGGATGGG CGAATTC
AGCGGAGGAA AAGAAACTAA CCAGG????? ???????
AAGGGGAGAA AAAAAAACAA CCCGG????? ???????
AGGGGAGGAA AAAAAACCAA CCCGG????? ???????
A????????? ?????????? ?????????? ???????
AGCGGAGGAA AAGAAACTAA CCAGGAAGGG CGAATTC
AGCGGAGGAA AAGAAACTAA CCAGGAAGGG C??????
AGCGGAGGAA AAGAAACTAA CCAGG????? ???????
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Appendix C

***Phlebotomus alexandri* ITS2 sequence alignment:**

Sequences with more than 300 base pairs

```
12      538
IZ53I     AATTCGCCCT TCGCAGCTAA CTGTGTGAAA TCGTGTGAAC TGCAGGACAC
IZ63I     AATTCGCCCT TCGCAGCTAA CTGTGTGAAA TCGTGTGAAC TGCAGGACAC
IZ56I     AATTCGCCCT TCGCAGCTAA CTGTGTGAAA TCGTGTGAAC TGCAGGACAC
P.alex    ?????????? ??CGCAGCTA ACTGTGTGAA ATCGTGTGAA CTGCAGGACA
TK07I     ?????????? ?????????? ?????????? ?????????? ??????????
TK09I     ?????????? ?????????? ?????????? ?????????? ??????????
IZ73I     ?????????? ?????????? ?????????? ?????????G GCTGCGGGAC
IZ60I     AATTCGCCCT TCGCAGCTAA CTGTGTGAAA TCGTGTGAAC TGCAGGACAC
IZ52I     ?????GCCCT TCGCAGCTAA CTGTGTGAAA TCGTGTGAAC TGCAGGACAC
P.dub     ?????????? ??CGCAGCTA ACTGTGTGAA ATCGTGTGAA CTGCAGGACA
IZ29I     ?????????? ?????????? ?????????? ?????????? ??????????
IZ75I     ?????????? ?????????? ?????????? ?????????? ??????????

ATGAACATCG ACATTTT-GA ACGCATATTG CGGTCCATGC AAAAGTTTAA
ATGAACATCG ACATTTT-GA ACGCATATTG CGGTCCATGC AAAAGTTTAA
ATGAACATCG ACATTTT-GA ACGCATATTG CGGTCCATGC AAAAGTTTAA
CATGAACATC GACATTTTGA ACGCATATTG CGGTCCATGC AAAAGTTTAA
??????CTCG AC-TTTT-GA ACGCATATTG CGGTCCATGC AAAAGTTTAA
??????CTCG ACATTTT-GA ACGCATATTG CGGTCCATGC AAAAGTTTAA
CTGAAC-TCG AC-TTTT-GA ACGCATATTG CGGTCCATGC AAAAGTTTAA
ATGAACATCG ACATTTT-GA ACGCATATTG CGGTCCATGC AAAAGTTTAA
ATGAACATCG ACATTTT-GA ACGCATATTG CGGTCCATGC AAAAGTTTAA
CATGAACATC GACATTTTGA ACGCATATTG CGGTCCATGC AAAAGTTTAA
?CTGAACTCG ACATTTT-GA ACGCATATTG CGGTCCATGC AAAAGTTTAA
???GAACTCG AC--TTTTGA ACGCATATTG CGGTCCATGC AAAAGTTTAA

ACTTGTTT-- AAACTGCATG GACCACGTAT GGTTGAGTAT CGTAAATATT
ACTTGTTT-- AAACTGCATG GACCACGTAT GGTTGAGTAT CGTAAATATT
ACTTGTTT-- AAACTGCATG GACCACGTAT GGTTGAGTAT CGTAAATATT
ACTTGTTT-- AAACTGCATG GACCACGTAT GGTTGAGTAT CGTAAATATT
ACTTGTTT-- AAACTGCATG GACCACGTAT GGTTGAGTAT CGTAAATATT
ACTTGTTT-- AAACTGCATG GACCACGTAT GGTTGAGTAT CGTAAATATT
ACTTGTTT-- AAACTGCATG GACCACGTAT GGTTGAGTAT CGTAAATATT
ACTTGTTT-- AAACTGCATG GACCACGTAT GGTTGAGTAT CGTAAATATT
ACTTGTTT-- AAACTGCATG GACCACGTAT GGTTGAGTAT CGTAAATATT
ATTTTTTTTT AAACTGCATG GACCACGTAT GGTTGAGTGT CGTAAATATT
ACTTGTTT-- AAACTGCATG GACCACGTAT GGTTGAGTAT CGTAAATATT
ACTTGTTT-- AAACTGCATG GACCACGTAT GGTTGAGTAT CGTAAATATT

AAGCAATTGA AATTGTTTTC TTTTT----- ----A-ATGA ACATTGGAGC
AAGCAATTGA AATTGTTTTC TTTTT----- ----A-ATGA ACATTGGAGC
AAGCAATTGA AATTGTTTTC TTTTT----- ----A-ATGA ACATTGGAGC
AAGCAATTGA AATTGTTTTT TTATTTTTTT TTCTAAAAAA ACATTGGAGC
AAGCAATTGA AATTGTTTTT TATTTTTTTT TTATAAAAAA ACATTGGAGC
AAGCAATTGA AATTGTTTTT TATTTTTTTT TTATAAAAAA ACATTGGAGC
AAGCAATTGA AATTGTTTTC TTTTT----- ----A-ATGA ACATTGGAGC
AAGCAATTGA AATTGTTTTC TTTTT----- ----A-ATGA ACATTGGAGC
AAGCAATTGA AATTGTTTTC TTTTT----- ----A-ATGA ACATTGGAGC
AAGCAATTGA AATTGTTTTC TTTTGT---- ------AA-A ACATTGGAGC
AAGCAATTGA AATTGTTTTC TTTTT----- -----AATGA ACATTGGAGC
AAGCAATTGA AATTGTTTTC TTTTT----- -----AATGA ACATTGGAGC
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TATGGAAAAT AATTTTTTCA TGCTCTTAAT ATA--TTGTT TTAAAGCATA
TATGGAAAAT AATTTTTTCA TGCTCTTAAT ATA--TTGTT TTAAAGCATA
TATGGAAAAT AATTTTTTCA TGCTCTTAAT ATA--TTGTT TTAAAGCATA
TATGGAAATT AATTTTTTCA TGCTCTTAAT ATATATATTT TTAAAGCATA
TATGGAAATT AATTTTTTCA TGCTCTTAAT ATATATATTT TTAAAGCATA
TATGGAAATT AATTTTTTCA TGCTCTTAAT ATATATATTT TTAAAGCATA
TATGGAAAAT AATTTTTTCA TGCTCTTAAT ATA--TTGTT TTAAAGCATA
TATGGAAAAT AATTTTTTCA TGCTCTTAAT ATA--TTGTT TTAAAGCATA
TATGGAAAAT AATTTTTTCA TGCTCTTAAT ATA--TTGTT TTAAAGCATA
TATGGAATAA GTATTTTTCA TGCTCTTAAT ATA--TATAT TTTAAGCACA
TATGGAAAAT AATTTTTTCA TGCTCTTAAT ATA--TTGTT TTAAAGCATA
TATGGAAAAT AATTTTTTCA TGCTCTTAAT ATA--TTGTT TTAAAGCATA

TTTGAATGTA CCCGATATAT AAATATTAAA AAAAAAATAA --TATAATAA
TTTGAATGTA CCCGATATAT AAATATTAAA AAAAAAATAA --TATAATAA
TTTGAATGTA CCCGATATAT AAATATTAAA AAAAAAATAA --TATAATAA
TTTGAATGTA CCCGATATAT AAATATTAAA AA----AAAT ---A---TAA
TTTGAATGTA CCCGATATAT AAATATTAAA AAAAATATAA ----------
TTTGAATGTA CCCGATATAT AAATATTAAA AAAAATATAA ----------
TTTGAATGTA CCCGATATAT AAATATTAAA AAAAAAAAAA A-TATAAAAG
TTTGAATGTA CCCGATATAT AAATATTAAA AAAAAAATAA --TATAATAA
TTTGAATGTA CCCGATATAT AAATATTAAA AAAAAAATAA --TATAATAA
TTTGAATGTA CCCAATATAT AA-TATTAAA AAGATGAAAT -----A-TAA
TTTGAATGTA CCCGATATAT AAATATTAAA AAAAAAAAAA AATATAATAA
TTTGAATGTA CCCGATATAT AAATATTAAA AAAAAAATAA --TATAATAA

GTGGTATATC AAAGTCAT-G GATAATTTTT T--CTATAAA AATTTGAAAA
GTGGTATATC AAAGTCATTG GATAATTTTT T--CTATAAA AATTTGAAAA
GTGGTATATC AAAGTCATTG GATAATTTTT T--CTATAAA AATTTGAAAA
GTGGTATATC AAAGTCATTG GATAATTTTT TT-CTATAAA AATTTGAAAA
GGGGTATATC AAAGTCATTG GATAATTTTT TTTCTATAAA AATTTGAAAA
GGGGTATATC AAAGTCATTG GATAATTTTT TTTCTATAAA AATTTGAAAA
GGGGTATATC AAAGCCTTTG GAAAATTTTT T--CTATAAA ATTTTGAAAA
GTGGTATATC AAAGTCATTG GATAATTTTT T--CTATAAA AATTTGAAAA
GTGGTATATC AAAGTCATTG GATAATTTTT T--CTATAAA AATTTGAAAA
GTGGTATATC ATAGTCATTG AATGATTT-- -------AAA AATCTTAAA-
GGGGTATATC AAAGTCTTTG GAAAATTTTT T--CTATAAA AATTTGAAAA
GTGGTATATC AAAGTCATTG GATAATTTTT T--CTATAAA AATTTGAAAA

AGACATACTG TTATACATAA AAGCTTATTT ATTTATTTTT TTTTTTAATA
AGACATACTG TTATACATAA AAGCTTATTT ATTTATTTTT TTTTT-AATA
AGACATACTG TTATACATAA AAGCTTATTT ATTTATTTTT TTTTA--ATA
AGACATATTG TTATACAGAA AAGCTTAT-- --TTTTTTTT ------AATA
AAACATATTG TTATACAAAA AAGCTTATTT TTT--TTTT- -------AAA
AAACATATTG TTATACAAAA AAGCTTATTT TTT--TTTA- -------AAA
AAACTTCCGG TTATACATAA AACCTTATTT TTTTTTTTTT TTTTT-AAAA
AGACATACTG TTATACATAA AAGCTTATTT ATTTATTTTT TTTTT-AATA
AGACATACTG TTATACATAA AAGCTTATTT ATTTATTTTT TTTTT-AATA
-GACATATTG TTATACAGAA AAACTTATAT CTTTTTTTTT -------AAT
AAACATACGG TTATACATAA AAGCTTATTT TTTTTTTTTT TTTTT-TAAT
AGACATACTG TTATACATAA AAGCTTATTT ATTTATTTTT TTTTT-AATA

TAAAAGGGAT TATT-CACTA TAAAAAATAT GCTAAAGAAA AAAAAAA---
TAAAAGGGAT TATT-CACTA TAAAAAATAT GCTAAAGAAA AAAAAAA---
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TAAAAGGGAT TATT-CACTA TAAAAAATAT TCTAAAGAAA AAAAAAAAAA
TAAAAGGGAT TATT-CACTT TAAAAAATAT GCACAAAAAA AAACTTA--A
AAAAAGGGGT TTTTTCCTTT TAAAAAATTG GCCCAAAAAA AAATTTA---
AAAAAGGGAA TTATCCCTTT AAAAAAAAAG GCCAAAAAAA AACTTTA---
AAAAAGGGAT TTTT-CCCTA TAAAAAATTT GCTAAA-AAA AAAAAAAAAA
TAAAAGGGAT TATT-CACTA TAAAAAATAT GCTAAAGAAA AAAAAAAAAA
TAAAAGGGAT TATT-CACTA TAAAAAATAT GCTAAA-GAA AAAAAAAAA-
AAAAAGGGAT TATT-CAATA TATATAATGT GCAAAAATAT AAAATTAT-A
AAAAAGGGGT TTTTTCCCTA AAAAAAATTT GCAAAAAAAA AAAAAA????
TAAAAGGGAT TATT-CCCTA TAAAAAATAT GCTAAAAAAA AAAAAAAA??

TTGCGATCTC AACTCATACG TGACTACCCC CTGAATTTAA GCATATTAAT
TTGCGATCTC AACTCATACG TGACTACCCC CTGAATTTAA GCATATTAAT
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TTGCGATCTC AACTCATACG TGACTACCCC CTGAATTTAA GCATATTAAT
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AAGCGGAGGA AAAGAAACTA ACCAGGAAGG GCGAATTC
AAGCGGAGGA AAAGAAACTA ACCAGGAAGG GCGAATTC
AAGCGGAGGA AAAGAAACTA ACCAGGATGG GCGAATTC
AAGCGGAGGA AAAGAAACTA ACCAGG???? ????????
AAAGGGGAGA AAAAAAAACA ACCCGG???? ????????
AAGGGGAGGA AAAAAAACCA ACCCGG???? ????????
AA???????? ?????????? ?????????? ????????
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AAGCGGAGGA AAAGAAACTA ACCAGGAAGG GC??????
AAGCGGAGGA AAAGAAACTA ACCAGG???? ????????
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